REVISED DRAFT DETAILED REVIEW PAPER

FOR

AVIAN TWO-GENERATION TOXICITY TEST

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LESLIE TOUART, PH.D.
WORK ASSIGNMENT MANAGER
U.S. ENVIRONMENTAL PROTECTION AGENCY
ENDOCRINE DISRUPTOR SCREENING PROGRAM
WASHINGTON, D.C.

BATTELLE
505 King Avenue
Columbus, Ohio 43201

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SPECIAL NOTE

A substantial technical contribution to the creation of this document was provided by Dr. Ann Fairbrother. Written text included the author's own published work, either as a lead or co-author. During this time, Dr. Fairbrother was working for a private company. She has since taken a position with the EPA.

1.0 EXECUTIVE SUMMARY

Endocrine disruptors are any chemicals that are known or suspected to cause adverse endocrine effects in organisms or their progeny. Such chemicals have received increased attention over the past decade because of the potential harm they can do to wild and domestic animals and ultimately to humans. Therefore, Congress authorized the United States Environmental Protection Agency (EPA) to develop a program to screen a wide array of chemicals found in drinking-water sources and food to determine whether they possess estrogenic or other endocrine activity that could have disruptive endocrine effects in humans. The aim of this program is to develop a two-tiered approach: that is, a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1), and a set of *in vivo* tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial chemicals, and environmental contaminants. The organisms used in the screening and testing will represent a variety of taxonomic groups, such as fish, birds, and mammals, for example.

The present detailed review paper fulfills one of EPA's objectives in its validation process, namely, to summarize, explain, and document the relevant principles, methods, and techniques for a Tier 2 multigenerational test to determine the effects of potential endocrine-disrupting chemicals on *birds*. Although avian species are not part of the Tier 1 screening battery for assessment of the effects of endocrine-disrupting chemicals, they are included in Tier 2. Birds are fundamentally different from mammals in the control of their sexual differentiation, and therefore, mammalian tests provide little predictive value for assessing a chemical's potential impact to birds; separate testing is required.

The present report contains a review of the current literature, and a recommendation of an initial Tier 2 protocol and of an organism that will best meet the needs for testing. It identifies issues that could require prevalidation studies. The recommended avian test protocol is designed to accomplish the following:

- determine whether effects are a primary or secondary disturbance of endocrine function
- establish exposure, concentrations, timing, and effects relationships
- be sensitive and specific
- assess relevant endpoints
- include a dose range for full characterization of effects
- be conducted in accordance with good laboratory practices (GLP)
- be validated.

Two quail species, the northern bobwhite, *Colinus virginianus*, and the Japanese quail *Coturnix japonica*, were considered for the test species, because they are commercially cultured and available year-round, are among the few avian species that breed successfully under laboratory conditions, and unlike most birds, are able to produce eggs almost indefinitely under long-day photoperiods. Also, both species are accepted models for assessing acute and reproductive effects of pesticides and other chemicals in birds. Although the Japanese quail is not indigenous

to the United States and has undergone extensive domestication, and therefore may be less representative of wild species, it is recommended here as the preferred test species because of its small size, high fecundity, well-characterized reproductive biology, and in particular, its very rapid incubation and maturation stages. Pragmatically, the completion of a mutigenerational test using Japanese quail can be completed in less than half the time of a test using the bobwhite.

The experimental design of avian reproduction tests has undergone considerable discussion. The tests can be designed to define concentrations of environmental chemicals at which no observable adverse effects occur (NOAECs), or they can be designed to develop dose-response relationships for endpoints of concern (ECx). Two principal exposure protocols have also been considered for the parental (P1) generation in the avian two-generation reproduction test; a "proven breeder design," in which reproduction is monitored pre-exposure, and a "pre-egg laying exposure design," in which effects on maturation are included. Several first-generation (F1) exposures have also been considered, the two most likely of which are treatment from hatch through egg laying and no treatment. Currently, there are insufficient data to determine the combination of exposure protocols for the P1 and F1 generations that is the most robust for documenting changes in ecologically important fitness endpoints, and that at the same time is most effective in determining mechanism of action. A side-by-side performance evaluation in prevalidation of the prebreeding and proven-breeder exposure regimens and the F1 treatment options is recommended to evaluate the sensitivity and cost-benefit of these exposure protocols.

Because of the variety of responses that test substances can induce, a broad set of reproductive fitness and physiological endpoints was selected to evaluate reproductive impact and endocrine activity in an avian two-generation reproduction toxicity test. Emphasis was given to endpoints recommended by the Endocrine Disruptor Screening and Testing Advisory Committee and the Organization for Economic Cooperation and Development (Bennett, et al., 2001). Additional endpoints were added from the literature review. Fitness endpoints are egg production and viability, hatching success, survival of chicks to 14 days of age, genetic sex, onset of sexual maturation, cloacal gland area, body weight, and male copulatory behavior. The physiological endpoints are gross morphology and histology of specific organs, levels of sex hormones, corticosterone, and thyroid hormones by specific methods. Because of the effects of handling on plasma concentrations of many of these hormones, it is preferable that hormone status be evaluated via the noninvasive sampling of fecal/urate matter. Monitoring of T4/T3 values over time would particularly benefit from sampling of feces; however, methods for fecal analysis have not been developed for these hormones, although they are excreted in the bile. Measuring the sperm:egg interaction is recommended as an inexpensive, but direct means of assessing gender-specific effects on fertility during egg production and for establishing relative fertility of proven-breeder males prior to treatment. Some modification of these endpoints is recommended to reduce redundancy, increase the cost-effectiveness of the test, and provide higher-quality data.

To further optimize two-generation testing with Japanese quail, several research areas have been identified:

- Many strains of Japanese quail have been developed, largely along egg production or body mass lines. The impact of strain selection on the ability of the test to detect endocrine activity and reproductive deficit needs to be evaluated.
- Japanese quail are recommended over the northern bobwhite largely for pragmatic reasons. Information that is not known at this time that could greatly influence this selection is the relative sensitivity of these two species to reproductive and endocrine effects of environmental chemicals.
- The potential impact of natural phytoestrogens in the feed on the outcome the test needs to be delineated so that limits of contamination can be set.
- If ANOVA methods continue to be applied to avian reproduction toxicity tests, a statistical approach for delayed effects must be investigated.
- Though seemingly a minor data gap, the lack of specific information on husbandry requirements of the Japanese quail that will result in consistent results in laboratory toxicity tests is important.
- The number of replicates (pens) needed for dose-response testing is not known. The replicate needs for sufficient power to detect an effect in the various physiological endpoints using ANOVA approaches have not been established.
- The most appropriate treatment regimen needs to be defined for the P1 and F1 generations to best characterize effects of potential endocrine disruptors on fitness endpoints with consideration of endocrine and other toxic effects.
- Baseline data on the variance, sensitivity, and range of responses of many of the selected and potential physiological endpoints (steroid and thyroid hormones, behavior, vitellogenin, very low density lipoproteins, aromatase) are needed.
- The source of the *in ovo* concentrations of test substances has impact on decisions regarding exposure duration and interpretation of endpoint responses. However, chemical transfer to the egg has been variably described from tissue or dietary sources. Elucidation of source transfer is needed.
- Further study of estrogenic xenobiotics that require metabolic activation is needed to determine whether or not this occurs in the embryo and to understand its relationship to this sensitive developmental period.

Considerations for a recommended protocol are discussed in Section 12 of this DRP using the Japanese Quail as the test system.

2.0 INTRODUCTION

2.1 DEVELOPING AND IMPLEMENTING THE ENDOCRINE DISRUPTOR SCREENING PROGRAM (EDSP)

Chemicals that are known or suspected of being endocrine disruptors (Kavlock et al. 1996), also referred to as hormonally active agents (NRC 1999), have received increased attention over the past decade. In 1996, the passage of the two laws, the Food Quality Protection Act (FQPA) and Amendments to the Safe Drinking Water Act (SDWA) mandated the United States Environmental Protection Agency (EPA) to screen substances found in drinking water sources or food to determine whether they possess estrogenic or other endocrine activity (Federal Register 1998a, 1998b). Pursuant to this goal, the EPA is required to "develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or other such endocrine effect..." (FQPA 1996).

In 21 U.S.C. §346a(p)(3), the FQPA also states that in carrying out its screening program, the EPA

> (A) shall provide for the testing of all pesticide chemicals and (B) may provide for the testing of any other substance that may have an effect that is cumulative to an effect of a pesticide chemical if the Administrator determines that a substantial population may be exposed to such a substance.

Additionally, Congress amended the Safe Drinking Water Act (SDWA) (42 U.S.C. §300j-17), authorizing the EPA

> to provide for the testing, under the FFDCA Screening Program . . . any other substance that may be found in sources of drinking water if the Administrator determines that a substantial population may be exposed to such substance.

Prior to the passage of the FQPA and the SDWA, the EPA initiated several endocrine disruptor investigations, including the development of a special report and effects assessment (EPA 1997a); a series of endocrine disruptor methods workshops funded by the World Wildlife Fund, Chemical Manufacturers Association (later known as the American Chemistry Council), and the EPA (Gray et al. 1997; EPA 1997b; Ankley et al. 1998); and co-sponsorship (with the National Institute of Environmental Health Sciences [NIEHS] and the Department of the Interior) of an independent critical literature analysis of hormone-active toxicants in the environment by the National Academy of Sciences (NRC 1999).

The EPA established the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), to provide recommendations regarding a strategy for developing a testing paradigm for compounds that may have activities similar to naturally-occurring hormones. Following the recommendations made by EDSTAC in its final report (EDSTAC 1998), the EPA established the

Endocrine Disruptor Screening Program (EDSP). The program's aim is to develop a two-tiered approach, e.g. a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial substances, and environmental contaminants (Federal Register 1998a, 1998b).

To date, the EPA has implemented the program on two fronts: (1) the development of the Endocrine Disruptor Priority Setting Database, and the approach that will be used to establish priorities for screening compounds, and (2) prevalidation and validation studies of some of the Tier 1 and Tier 2 assays that are likely to be included in the testing battery. The Endocrine Disruptor Methods Validation Subcommittee (EDMVS) has been set up to advise and review new and ongoing work in the validation of these assays.

The EDSP's proposed statement of policy, including public comments, was reviewed by a joint panel of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP) and the EPA Science Advisory Board (SAB) in May 1999. Gray et al. (1997), EDSTAC (1998), and the National Research Council (NRC 1999) concluded that a tiered approach relying on a combination of *in vivo* and *in vitro* screens for Tier 1 was scientifically reasonable. This conclusion was based upon each group's assessment of the current state of the science on the evaluation of agents affecting the endocrine system. Another consistent conclusion was the need to validate the individual screens and tests in the EDSP. Validation and peer review are prerequisites to the development and approval of test guidelines for regulatory use. Many of the documents cited above and other EPA EDSP-related information may be found at http:\\www.epa.gov/scipoly/oscpendo.

In addition to the EPA's domestic EDSP validation program, a separate effort to validate certain screening assays and tests for international use is being conducted by the Organization for Economic Cooperation and Development (OECD) Test Guidelines Program. The EPA actively participates as a member of the OECD test guidelines program and its Endocrine Disruptor Testing and Assessment Task Force. The EPA is relying on the OECD effort to serve as the mechanism for validation of some of the components of its EDSP. Separate domestic and international activities are necessary in that laws and regulatory procedures differ in various countries. Although international activities are distinct from domestic activities, overlapping membership on various committees ensures appropriate liaison and communication, eliminates duplication of effort, and facilitates international harmonization.

2.2 THE VALIDATION PROCESS

The EPA (and EDMVS) chose to follow the validation process established by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), of which the EPA was a charter member, for validation of the EDSP screening and testing methods. ICCVAM was established by the National Institute of Environmental Health Sciences (NIEHS) as a standing interagency committee to aid in the validation, acceptance, and harmonization of test methods designed to reduce animal use, refine procedures involving the use of animals so

that they would experience less stress, and to replace animal tests whenever appropriate (ICCVAM 2000). To this end, ICCVAM defined a flexible, adaptable framework for test method validation that was applicable to conventional and alternate methods, and could be applied to the needs of different agencies and regulatory processes.

The purpose of the validation is to establish the reliability and relevance of a test method with respect to a specific use. The process is science-driven, and addresses the scientific principles of objectivity and experimental design (NIEHS 1997). In addition, as stated in the ICCVAM report, "A test is considered validated when its performance characteristics, advantages, and limitations have been adequately determined for a specific purpose." (NIEHS 1997).

The validation process consists of four discrete phases: (1) initial protocol development, (2) prevalidation studies, (3) validation studies, and (4) external scientific peer review. The initial protocol, developed from existing information and experience (past and current research), serves as the starting point for initiating the validation process. Prevalidation studies consist of further development and optimization of specific initial protocols through targeted investigations. Either before or during prevalidation, a detailed review paper (DRP) addressing all critical areas outlined in *Validation and Regulatory Acceptance of Toxicological Test Methods* (NIEHS 1997) is prepared for each method to summarize, explain, and document decisions regarding the relevant principles, methods, and techniques recommended for the initial protocol. Targeted prevalidation investigations are designed to address questions necessary for completing an optimized, transferrable protocol suitable for interlaboratory validation studies. Validation studies consist of comparative interlaboratory studies to establish the reliability and relevance of the protocols developed in the prevalidation stage. Validation requires the development of a detailed review paper to document what is known about the assay system proposed for validation.

A test is considered validated when its performance characteristics, advantages, and limitations have been adequately determined for a specific purpose. The measurement of a test's reliability and relevance are independent stages in the validation of a test method, and both are required. Reliability is an objective measure of a method's intra- and interlaboratory reproducibility. If the test is not sufficiently reliable, it cannot be used for its intended purpose. Alternatively, if the test is not relevant, of questionable relevance to the biological effect of interest, or if it is not an appropriate measure of the effect, its reliability is academic. The relevance of a test may be linked to the mechanism of the toxic effect it measures and to its proposed uses (NIEHS 1997). The studies conducted will be used to develop, standardize, and validate methods, prepare appropriate documents for peer review of the methods, and develop technical guidance and test guidelines in support of the EDSP.

Following the validation studies, results of an external scientific peer review of the study and the optimized protocols will be used to develop the EPA test guidelines.

2.3 Purpose of the Review

The purpose of this detailed review paper (DRP), prepared as part of Work Assignment 2-16, is to define the basis and purpose of the proposed avian two-generation reproductive and developmental toxicity study for evaluating effects of potential endocrine-disrupting chemicals. The DRP will summarize, explain, and document the relevant principles, methods, and techniques; it will recommend an initial Tier 2 protocol that will best meet the needs for testing; and it will identify issues that could require prevalidation studies.

2.4 Objectives of the Avian Two-Generation Toxicity Study

Tier 2 is the final phase of the screening and testing program and therefore should provide more detailed information regarding the endocrine disruption activity of a tested chemical or mixture. To fulfill this purpose, tests are often longer-term studies designed to encompass critical life states and processes, a broad range of doses, and administration by relevant route of exposure. In addition, the effects associated with endocrine disrupting chemicals (EDCs) can be latent and not manifested until later in life or may not be apparent until reproductive processes occur in an organism's life history. Thus, tests for endocrine disruption often encompass two generations to address effects on fertility and mating, embryonic development, sensitive neonatal growth and development, and transformation from the juvenile life state to sexual maturity. The results form the Tier 2 testing should be conclusive in documenting a discernable cause-and-effect relationship of chemical exposure to measurable manifestation in the test organisms.

The avian test protocol that will be recommended in this report (Section 11.0) will be designed to be capable of the following:

- to determine whether effects are a primary or secondary disturbance of endocrine function
- to establish exposure, concentrations, timing, and effects relationships
- to be sensitive and specific
- to assess relevant endpoints
- to include a dose range for full characterization of effects
  to be conducted in accordance with good laboratory practices (GLP)
- to be validated.

Although avian species are not part of the Tier 1 screening battery, they are included in Tier 2 and serve an important role in that birds are fundamentally different from mammals in the control of sexual differentiation, and results from mammalian tests provide little predictive value for assessing a chemical's potential impact on birds. The following sections describe the methods used and results obtained from conducting this DRP on avian multigenerational test methods.

2.5 Methods Used in This Analysis

In Appendix A, a detailed description of the methods employed for the literature search, such as key words, databases, and results, is provided. After key papers were identified, retrieved, and read for content, pertinent information was synthesized to create this DRP. At the back of this report is a compact disk read-only memory (CD ROM) containing the Reference Manager Database of all documents reviewed. This database includes the reference citation and abstract.

2.6 Acronyms and Abbreviations

Table 2-1 lists the acronyms and abbreviations used in the DRP, with the exception of commonly used units, such as h for hour or L for liter. Each of the acronyms and abbreviations also is introduced at first use in the text.

Table 2-1. Acronyms and Abbreviations

ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
A&M	(Texas) Agricultural and Mechanical (University)
ASTM	American Society for Testing and Materials
apo	apolipoprotein
BSA	bovine serum albumin
BST	bed nucleus of the stria terminalis, part of the quail brain
CNS	central nervous system
CV	coefficient of variation
CyA	cyproterone acetate
DDE	dichlorodiphenylethylene
DDT	dichlorodiphenyltricloroethane
DES	diethylstilbestrol
DEHP	diethylhexylphthalate
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
DRP	detailed review paper
EB	estradiol benzoate
EC50	median effective concentration
ED*x*	effective dose (*x* = percentage of effect, from 0 to 100)
EDC	endocrine-disrupting chemical
EDMVS	Endocrine Disruptor Methods Validation Subcommittee
EDSP	Endocrine Disruptor Screening Program
EDSTAC	Endocrine Disruptor Screening and Testing Advisory Committee
EDSTP	Endocrine Disruptor Screening and Testing Protocols
EE_2	ethinyletradiol
ELISA	enzyme-linked immunosorbent assay
EPA	U.S. Environmental Protection Agency
EROD	ethoxyresorufin-O-deethylase
FFDCA	Federal Food, Drug, and Cosmetics Act
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FQPA	Food Quality Protection Act
FSH	follicle-stimulating hormone
GLP	good laboratory practice
GnRH	gonadotropin releasing hormone

Table 2-1. Acronyms and Abbreviations (Continued)

HAH	halogenated aromatic hydrocarbon
HCP	highly carboxylated porphyrine
hCG	human chorionic gonadotropin
HPG	hypothalmic-pituitary-gonadal
HPV	high production volume
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
in vitro	outside the body, in an artificial environment
in ovo	in the egg
in vivo	within the body
IPVL	inner perivitelline layer
LC50	median lethal concentration
LH	luteinizing hormone
LOAEL	lowest observable adverse effect levels
MTD	minimum tolerated dose
NIEHS	National Institute of Environmental Health Sciences
NOAEL	No observable adverse effect level
NRC	National Research Council
OC	organochlorine
OECD	Organization for Economic Cooperation and Development
OP	organophosphorus
OPPT	Office of Pollution Prevention and Toxics, EPA
OPVL	outer perivitelline layer
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
P4	progesterone
PMSG	pregnant mares' serum gonadotropin
POM	medial preoptic nucleus, part of the quail brain
Prl	prolactin
Random-bred	Random-bred lines are relatively large populations of birds (>100) in which minimal selection of breeding stock is done by the curator (Pisenti et al. 1998)
RH	relative humidity
RIA	radioimmunoassay
RMD	Reference Manager Database
RNA	ribonucleic acid
RTI	Research Triangle Institute
SAB	Science Advisory Board
SAP	Scientific Advisory Panel
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide electrophoresis
SDWA	Safe Drinking Water Act
SHBG	sex hormone binding globulin
T	testosterone
T1S	Tier 1 Screening
T3	triiodothyronine
T4	thyroxin
TCDD	tetrachlorodibenzodioxin
TSH	thyroid-stimulating hormone
UBC	University of British Columbia
USC	United States Code
VTG	vitellogenin

3.0 OVERVIEW AND SCIENTIFIC BASIS OF AVIAN TWO-GENERATION TESTS

Effects of chemicals on avian reproduction, development, and survival have been studied for several decades, and standardized protocols have been in place for screening potential environmental contaminants for such effects. Although these methods have some relevance to the discrimination of endocrine disruptors from among the category of all substances that causes adverse effects, they are not sufficiently robust to determine mode of action. Because endocrine disruptors are categorized on the bases of how they exert adverse effects in the animal, specific endpoints must be built into the studies to differentiate them from other "reproductive or developmental toxicants" (DeFur et al. 1999). Furthermore, current test protocols are not designed to determine long-term effects of *in ovo* exposure, which may be the most relevant responses to endocrine-disrupting compounds in birds (Fry 1995). Therefore, a two-generation avian reproduction study is being developed to provide the necessary protocol to determine endocrine disruption potential and ecologically relevant effects of environmental chemicals.

Endocrine disruptors are "exogenous agent[s] that interfere with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (Kavlock et al. 1996). Such effects must result in adverse health effects in an organism or its progeny, to be ecologically relevant (DeFur 1999). It is important to note, however, that endocrine disruptors are defined on the basis of their mechanism of action. Many physiological processes that are hormonally regulated, including reproduction and development, may be affected by chemicals through other toxicological mechanisms. Hoffman (1990) reviewed the data on embryo toxicity and teratogenicity of environmental chemicals to bird eggs. Many chemicals have shown such effects, either through direct application or via maternal transfer, including organochlorines (OCs), polycyclic aromatic hydrocarbons (PAHs), organophosphorus compounds (OPs), some herbicides, and fungicides. However, there are multiple causes of such effects, not all of which are endocrine-related.

For example, selenium is a well-known avian embryotoxicant, causing severe embryonic developmental effects following *in ovo* exposure. Such effects are due to interference with glutathione peroxidase and subsequent oxidation of cell membranes (Ohlendorf 1996). Thus, selenium would be classified as a reproductive and developmental toxicant but not as an endocrine disruptor. Endocrine disruptors need to interact with the hormone system, not simply alter a process that is under normal endocrine control (DeFur 1999).

The concept that environmental chemicals have the potential to disrupt hormonally regulated processes in birds is not new. Such mechanisms have been studied since the late 1960s, and it was first discussed as a generalized class of chemicals at a NIEHS symposium, "Estrogens in the Environment," held in Eugene, Oregon, in 1979. Chemicals such as OCs, PAHs, diethystilbesterol (DES), and others (e.g., nonylphenols) were identified as having estrogenic properties due to competitive binding with cellular estrogen receptors (McLachlan 1997). Potential endocrine effects in birds exposed to environmental pollutants were reviewed further by Rattner et al. (1984), who introduced the concept of relative potency to the discussion of

environmental relevance. For example, whereas dichlorodiphenyltrichloroethane (DDT) and its derivatives have been shown to bind to nuclear estrogen receptors in the magnum and shell gland regions of the oviduct, their relative potency is only 1/1000 of that of the natural estrogen, 17β-estradiol. This further underscores the necessity of conducting studies in a dose-response fashion and correlating measured hormonal changes with ecologically relevant fitness endpoints, such as successful reproduction, growth, and maturation. Rattner et al. (1984) also broadened the discussion to include the potential for chemicals such as the OCs to result in thyroid dysfunction. The current definition of endocrine-disrupting chemicals now includes those that act or have the potential to act on estrogens, androgens, and thyroid hormone systems.

3.1 Avian Endocrinology

"The endocrine system is responsible for regulating most of the body's essential functions. It controls reproduction and secondary sex characteristics, molting and metamorphosis, fluid balances, growth rates, salt balance, and response to stress, among others. These functions are accomplished by a diverse set of small organs located throughout the body that communicate with each other and their target organs by releasing small amounts of very potent substances known as hormones. The primary endocrine organs are the adrenal glands, pituitary, thyroid, parathyroid, ovaries/testes, and pancreas. The kidney produces hormones in addition to filtering the blood. In mammals, the placenta also functions as an important endocrine organ Although many of the processes and hormones involved with reproduction, development, and homeostasis are well conserved across species, there are sufficient differences between mammalian and avian systems to warrant the development of avian-specific tests (Dawson 2000).

Control of the endocrine system is by the neurosecretory neurons within the hypothalamus. These receive input from internal and external cues and also control the rate at which the hypothalamus synthesizes and secretes various peptides or glycoproteins known as releasing hormones (e.g., gonadotrophic releasing hormone [GnRH]). These hormones then stimulate the pituitary to synthesize and secrete other peptide hormones (e.g., follicle stimulating hormone [FSH]; thyroid stimulating hormone [TSH]), which pass into the circulation and go to the target endocrine glands. Glands such as the ovaries, testes, and oviduct are stimulated to secrete the active steroid sex hormones, including estrogens and androgens. "Estrogen, testosterone, and other steroid hormones are distributed around the body bound to another protein, the sex hormone-binding globulin (SHBG). The relative affinity of the hormones to bind to SHBG versus to cell receptors influences the potency of hormones within and across species. Interaction of chemicals with the SHBG-hormone binding may be a cause of species-specific differences in hormone-mediated effects of xenobiotics. There are a number of interactions among the various endocrine axes and between the endocrine and nervous systems. In the case of the reproductive, hypothalamic-pituitary-gonadal (HPG) axis, steroid hormones produced by the gonads feed back to the hypothalamus and pituitary gland to alter HPG axis function. This is termed a negative feedback loop and is a mechanism by which many of the endocrine axes are regulated. Blood calcium levels are regulated in a manner similar by the parathyroid gland. Estrogen interacts with parathormone, which is produced by the parathyroid gland, regulating calcium levels by causing an increased deposition of calcium in the long bones. Hormones

produced by the kidney also participate in the regulation of calcium as well as in the maintenance of blood pressure and proper balance in the body of ions such as potassium and sodium. The adrenal glands, located just above the kidneys, produce corticosteroids, which are important under conditions of chronic stress" (Bennett, et al., 2001).

"The corticosteroids also influence the immune system and estrogen production. Less well known is their production of hormones that interact with the kidney to maintain ionic balance" (Bennett, et al., 2001).

"In birds, sex steroid hormones are responsible for a variety of reproductive functions. These include development of secondary sexual characteristics such as plumage coloration and mating songs, phenotypic gender determination including oviduct development and maturation, shell gland function, oviduct development and maturation, and cornification of the cloacal epithelium. Progesterone causes development of the brood patch, which is further vascularized under the influence of estrogen. Progesterone also is responsible for broody behavior, such as nest attentiveness during incubation and during posthatch in altricial species. Estrogen also regulates calcium deposition and release from bones, as well as brain development. In birds and other egg-laying animals, estrogen is responsible for the stimulation of production of vitellogenin (VTG) by the liver. VTG is the precursor for the primary proteins that are incorporated into the egg for nourishment of the embryo during prehatch development" (Bennett, et al., 2001).

The steroid sex hormones are synthesized from cholesterol through the hydrolysis of side chains by cytochrome P450 enzymes in families 11, 17, 19, 21, and 27, to form progesterone. These enzymes are not inducible by xenobiotics, but they can be inhibited. Progesterone is further modified to form testosterone and its active derivative, androstenedione. Another P450 enzyme, aromatase, takes these androgens to estrogens, estradiol and estrone, respectively (Fairbrother 2000). Cytochrome P450 enzymes also play a role in the degradative metabolism of the steroid hormones. Cytochrome P450 enzymes in families 1 through 4 metabolize natural steroids to more polar forms for excretion in the urine and are inducible by a variety of chemicals (Dawson 2000).

"The thyroid gland is another major endocrine organ. It is a butterfly-shaped gland located at the base of the neck of most vertebrates and depends upon an adequate supply of iodine for normal functioning" (Bennett, et al., 2001). Thyroid hormones are peptide hormones and are synthesized in the follicles of the thyroid gland; this synthesis produces a large globular glycoprotein called thyroglobulin. This contains iodinated tyrosine residues, which are coupled to form iodinated thyronine, the major form of which contains four iodides and is known as thyroxin, also known as tetraiodothyronine (T4). T4 is the major circulating hormone, but is not the active form. T4 is converted into tri-iodothyronine (T3), which is the physiologically active thyroid hormone. Conversion occurs in the liver and is controlled by the enzyme Type I deiodinase. Only about 30% to 40% of the circulating T4 is converted to T3; another 15% to 20% is deaminated to form tetraiodothyroacetate, which is rapidly excreted. Other glucuronides also are formed and excreted.

"Production of thyroid hormone is regulated very closely by the hypothalamus and pituitary, through a negative feedback cycle. The corticosteroids, testosterone, and estrogen can depress production of thyroid hormone, and some of the soluble mediators of immune system function (interferon and interleukins) have slight effects as well. T3 is responsible for controlling the metabolic rate of an animal, regulating body temperature, interacting with growth hormone to determine body size, and playing an integral role in molting, metamorphosis, and smoltification of various species of invertebrates, amphibians, and fish" (Bennett, et al., 2001).

3.2 Differences in Avian and Mammalian Endocrine Systems

"There are many similarities among birds, mammals, and other vertebrate classes regarding endocrine systems. These vertebrate classes share similar hormones and hormone receptors, and fundamental feedback mechanisms are similar. However, important differences between birds and mammals and other vertebrate classes do exist. Birds offer unique morphological, physiological, and behavioral adaptations that are not widely found in other vertebrates, including flight, which requires adaptations for high metabolic rate and reduced body mass, oviparity with hard-shelled eggs, a different physiological basis for gender development, and complex mate attraction behaviors, especially in songbirds" (Bennett et al. 2001). These differences were reviewed by Fry (1995) and Fairbrother (2000), and they are summarized here.

Sex determination and control of differentiation is linked to the heterogametic sex. In mammals, this is the male (XY); in birds, it is the female (ZW). The so-called default sex, which is the phenotype to which the embryo will develop in the absence of sex-specific hormones, is the homogametic sex. Mammals will develop as female (XX) and birds as male (ZZ) if no sex differentiation hormones are present. Therefore, birds require that estradiol be synthesized to cause differentiation of the gonad into an ovary. Lack of estrogen, regardless of androgen levels, will result in development of phenotypic males. In mammals, the reverse is true: embryos will develop into phenotypic females unless sufficient levels of androgens are present to induce gonadal differentiation into testicular tissue. In mammals, normal maternal estrogens are sequestered by fetoprotein, which has a high estrogen binding affinity and protects fetal tissues from estrogen exposure. Exogenous estrogenic substances also may bind to this protein, reducing the potential for effect on the developing embryo. Birds do not have this protein, and embryos are exposed *in ovo* to the same level of estrogen as was in maternal circulation during egg formation.

Because of the differences in steroid hormonal control of sexual differentiation, xenobiotic estrogens have different effects in birds and mammals during embryonic development. In male birds, excess estrogens will stimulate the primordial germ cells to become localized in the cortex of the gonad as well as in the more normal location in the medulla in a dose-dependent manner. These cortical cells differentiate into primordial follicles, and the gonad begins to resemble an ovary. The seminiferous tubules are retained, but the number of primordial germ cells will be lower than normal, resulting in low to no spermatogenesis. Genetic males also may develop an oviduct. Estrogenization of female birds does not change the ovary, but does change the oviduct and causes retention of the right oviduct, which in birds normally regresses prior to maturation.

Some of the OC pesticides and other xenobiotics need to be metabolized into active products before the estrogenizing effects are expressed. Most of the metabolic products of OCs are hydroxylated forms that are water-soluble and readily excreted in the urine/urates. However, in bird embryos, the metabolized products cannot be excreted (i.e., they remain in the egg), and so the embryo can be exposed throughout the length of gestation. In mammals, maternal excretion can reduce the effective dose and/or completely eliminate the product before the end of gestation. Because these products have 100 to 1000 times less potency or binding efficiency than natural estrogens, direct reproductive effects on adults are expected to be negligible. However, avian embryos are at significant risk because of retention in the egg and estrogen-dependency of sex expression.

3.3 Avian Two-Generation Test

The proposed avian two-generation reproductive study for determination of toxicants with endocrine-disrupting effects is based on a premise similar to that of the mammalian study (OECD 1999). The test must be sufficiently robust to document changes in fitness endpoints, such as reproductive output, developmental adequacy, and appropriate behaviors, but equally specific to determine mechanism of action. It is essential that a two-generation test be able to assess the impact of endocrine-disrupting chemicals on endocrine-mediated processes as systems organize during embryonic development and as they are activated in adult birds. There are four critical life stages of birds during which endocrine-mediated processes take place and that therefore could be sensitive to endocrine disruption: 1) *in ovo*, 2) offspring (F1) generation chick growth, 3) parental (P1) generation and F1 sexual maturation, and 4) P1 and F1 egg-laying. Therefore, the study must include two egg-laying cycles to assess effects on ecologically relevant fitness endpoints of endocrine dysfunction at each of these stages.

In brief, the test is designed to expose birds to environmental chemicals suspected as having endocrine-disrupting effects from examination of structure-activity relationships or test data from other species or *in vitro* studies. The P1generation is exposed, and reproductive endpoints such as egg production are measured, along with appropriate measures of estrogen, testosterone, and thyroid hormone synthesis, metabolism, and activity. The F1 generation is hatched and evaluated to 2 weeks of age, at which time a subset is selected for pairing and further evaluation of reproduction, development, and endocrine function. This generation may be exposed to the chemical throughout its lifetime, or a subset may be exposed while another cohort is not. The F2 generation also is hatched and observed to 2 weeks of age but is not exposed to the chemical. Test species will be either Japanese quail (*Coturnix japonica*) or bobwhite quail (*Colinus virginianus*) because of their small size, short time to sexual maturation, ease of handling, and an existing large database of information regarding reproductive effects of environmental contaminants.

The identification of endocrine-mediated effects on avian reproduction can occur at the test-individual or test-population level of analysis. This needs to be considered in determining the ultimate objective of the test. If the emphasis in a two-generation study is to determine the impact of a potential endocrine disruptor at the test-population level, then an integrative endpoint

that expresses the outcome of the study in terms of effect on overall productivity of a population must be identified. This type of study requires determination of dose-response curves for the integrative endpoint. Other, specific endocrine-related endpoints would then be evaluated alongside the primary productivity endpoint to identify the chemical as an endocrine disruptor rather than simply a reproductive toxicant, and to identify sensitive life stages. Endocrine-specific endpoints also may identify exposure levels at which endocrine-mediated effects occur in individual birds but not in the treatment group as a whole, or cause measurable hormonal changes without resulting in gross physiological differences. For example, because only 30% to 40% of the naturally circulating levels of T4 is converted into the active T3 form, a change in production of T4 may not result in significant effects to T3 levels. Thus, incorporation of sensitive measures of endocrine-related endpoints will allow a hazard assessment–that is, a determination of whether chemicals are potentially endocrine-disrupting–without resulting in ecologically relevant risk estimates (Bennett et al. 2001).

Treatment of the P1 generation can begin either prior to sexual maturation or after the onset of egg-laying. The advantages of starting dietary treatment prior to sexual maturation are ". . . the ability to measure changes in the onset of laying and effects resulting from inhibition or delay of gonadal development. The disadvantages are that birds that are incompatible or infertile for reasons other than the test substance cannot be removed, which may reduce the statistical power of the test and the ability to detect treatment effects if they exist. The advantages of starting treatment during egg-laying include the ability to remove nonlaying and infertile pairs prior to the initiation of treatment and the option to use pretreatment measurements as covariates in statistical analyses to remove nontreatment sources of variation. Starting treatment during egg-laying also provides information on rapidity with which reproductive effects can be observed, which can be useful in risk assessments of test substances that degrade rapidly in the environment. Because treatment effects on reproductive endpoints may increase during the course of the treatment period, statistical analyses need to be sensitive to the potential temporal patterns in the endpoint measurements" (Bennett, et al., 2001). Disadvantages include the inability to measure changes in the onset of laying and effects resulting from inhibition or delay of gonadal development. These two options are discussed in greater detail in Section 5.1.1.

Treatment of the F1 chicks ensures that endocrine-mediated effects occurring during growth and development of chicks as a result of direct exposure as well as from *in ovo* exposure will be assessed. Treatment of the F1 chicks also is considered to more closely represent a continuous-exposure scenario (Bennett, et al., 2001). "However, analysis and interpretation of the results will have to consider how effects occurring at various life stages may be confounded" (Bennett, et al., 2001). Additionally, effects from such full-life-cycle exposure scenarios need to be interpreted with caution for those test substances that degrade rapidly in the environment that could be present at only certain life stages. "Ending treatment of the F1 generation prior to egg-laying removes some of the potentially confounding effects of endocrine-mediated effects on F1 reproductive potential occurring from *in ovo* and/or early-life exposures. It can be argued that the effects of exposure during egg-laying can be observed in the P1 generation and may not need to be repeated in the F1 generation. However, by ending the treatment period just prior to egg-laying, there may be a temporal pattern of effects observed in the production of the F2

generation, depending on the clearance rate of the test substance and related deposition rate into eggs, and the rate of recovery of the F1 birds from any residual toxic effects" (Bennett, et al., 2001). Several exposure options have been proposed to cover these various arguments. They are discussed in more detail in Section 5.1.2.

The experimental design of avian reproduction tests has undergone considerable discussion. The tests can be designed to define concentrations of environmental chemicals at which no observable adverse effects occur, or they can be designed to develop dose-response relationships for endpoints of concern. Determination of no observable adverse effects levels (NOAELs) requires use of analysis of variance (ANOVA) designs, whereas development of dose-response curves uses regression techniques (Bennett et al. 2001). The issue of appropriate statistical design is examined in further detail in Section 5.4.2.

4.0 CANDIDATE TEST SPECIES

Two quail species, the northern bobwhite and the Japanese quail, are the most likely candidates for use in a two-generation reproductive screening assay and are the focus of this review. Because of their terrestrial habit, both species are considered to be representative of terrestrial birds and are accepted models for assessing both acute and reproductive effects of pesticides and other chemicals in wild birds (Office of Pollution Prevention and Toxics [OPPT], EPA, OPPT Guideline 850.230 [Federal Register 1978]; OECD Guideline 206 [OECD 1984]). Although both species, but particularly the Japanese quail, have undergone domestication and therefore may be less representative of wild birds, they are used in reproductive assays, because few wild species adapt well to laboratory conditions and breed successfully in the laboratory. Also, unlike most birds, the breeding cycle of quail is not restricted with a photorefractory period, so they are able to produce eggs almost indefinitely under photoperiods longer than about 12 h. In addition, as precocial species, they are likely to be more sensitive to changes in steroid concentrations during all life stages.

4.1 Japanese Quail (*Coturnix japonica*)

This Old World quail has been intensively domesticated for more than 800 years in Japan. Since the beginning of the twentieth century, breeding stock has been selected for body size and egg production throughout the world (Cooper 1976). Use of Japanese quail as a laboratory animal began in the late 1950s, and because of its adaptability to battery breeding cages, small size, and high fecundity, it has been used extensively in research. As a result, numerous strains of Japanese quail have been developed and conserved in government and academic institutions and by commercial suppliers. Among these strains, body size characteristics, rates of sexual maturation, and reproductive capabilities can differ significantly. The Japanese quail has been used extensively in reproductive toxicity testing in the European community and to a lesser extent in the United States. Some testing guidelines include detailed information on their laboratory husbandry (OECD Guideline 206; OPPT Guideline 850.2300; ASTM Method E1062-86).

4.1.1 Natural History

The Japanese quail, once classified as a race of the common quail (*Coturnix coturnix*), is now considered to form a superspecies with *C. coturnix* and possibly also with *C. pectoralis* (Cheng and Kimura 1990). It belongs to the largest family (Phasianidae) of the galliformes, and based on deoxyribonucleic acid (DNA)-DNA hybridization evidence is only distantly related to New World quail, such as the northern bobwhite (Hoyo et al. 1994). The Japanese quail is a migratory species widely distributed throughout eastern Asia. Feral populations of Japanese quail have been established in the Hawaiian Islands and in Great Britain, but three attempts to introduce the species as a game bird into the United States have been unsuccessful (Cooper 1976). Little habitat information is available for the Japanese quail; however, this ground-dwelling species has been observed using a wide range of open habitats, including cultivated areas. It feeds on a variety of plant materials and terrestrial invertebrates and nests in grassland areas. Clutch sizes vary from 5 to 8 eggs in Japan to 9 to 10 eggs in Russia. Incubation is typically 18 days in the wild (Hoyo et al. 1994). Siblings form coveys through their first winter and disperse the following spring. In the wild, their average body weight is about 90 g (Hoyo et al. 1994).

4.1.2 Availability, Culture, Handling

Several academic and governmental institutions in the United States and Canada maintain random-bred stocks of Japanese quail (Pisenti et al. 1999). The largest quail collection in North America is maintained at the Quail Genetic Stock Center at the University of British Columbia (UBC), Vancouver, British Columbia, through support by the Natural Sciences and Engineering Research Council of Canada. Among its conserved strains of Japanese quail are several outbred stocks including a wild type from Japan. In the United States, some of the oldest established quail lines for research are at the Universities of California, Georgia, and Maryland (Table 4-1). In addition, some commercial sources maintain large colonies of Japanese quail to supply research and toxicity testing organizations. Most of the stocks available in North America and Japan are derived from stocks originally selected for egg production and are smaller, faster-maturing, less-docile strains than those commonly used in the European community for reproductive toxicity testing. However, as the quail meat industry grows, more strains of the higher-body weight birds are being developed in the United States. As addressed in Section

4.1.3, the growth and reproductive differences between the strains can be substantial.

The care and handling of Japanese quail for laboratory use have been well documented. (NRC 1969; Cooper 1976; CCAC 1984; Ottinger and Rattner 1999), and testing guidelines include detailed information on their husbandry for reproductive toxicity tests (OPPT Guideline 850.230; OECD 206). *Coturnix* grow very rapidly; females reach sexual maturity in about 6 weeks, depending on strain. Day-old quail weigh about 7 g, and at maturity, they weigh between 120 g to more than 200 g. As a consequence, provision of adequate space per bird during this short growth period is crucial. During the growth period, temperature requirements that are 38ºC at hatch decrease by about 3ºC per week until the young reach 4 weeks of age. Young are very susceptible to drafts during this period. At 4 weeks of age, they are fully feathered and often are

moved to breeder cages. Pairing birds before sexual maturity aids in reducing aggressive behavior between pairs (NRC 1969). Early pairing can be accomplished, because gender is discernable by plumage color and pattern as early as 3 weeks of age. The cloacal gland, a bulbous structure located at the upper edge of the cloaca in males, also can be used to discriminate between sexes, but it is affected by photoperiod (Sachs 1967). Japanese quail are very hardy once the brooding stage is past.

Wild-type hens and hens from strains selected for egg production will lay about 1 egg per day and will produce about 300 eggs per year. Heavier strains lay fewer eggs (Table 4-2). The average weight of eggs laid by mature Japanese quail is about 10 g. However, older birds usually produce larger eggs, larger embryos, and larger chicks (Cooper 1976). A mating ratio of one male to one female results in the highest fertility rate. Duration of fertility after removal of the male is about 6 days. After remating the pair, fertile eggs are produced on the third day (Woodard and Abplanalp 1967). Full egg production is reached about 2 to 3 weeks after egg-laying begins. Optimal reproduction typically spans 5 to 6 months (CCAC 1993); maximum hatchability occurs when parents are between 8 and 20 weeks of age (CCAC 1984). Fertility in Japanese quail progressively decreases after about 56 weeks of age under stimulatory photoperiod (16L:8 D). By this time, courtship and mating behavior are greatly reduced and plasma testosterone levels also are decreased (Ottinger et al. 1983). Also, eggshells of older birds are usually thinner and result in a lower hatch (Cooper 1976).

Table 4-1. Stocks of Random-Bred or Wild Type Japanese Quail Maintained in North America and Europe[(a)]

Stock Name and Description	History of Origin	Number of Birds	Curator
Quail			
Random-bred, Arkansas RBS random-bred control from Eastern Shore random-bred	Acquired from Eastern Shore as random-bred control, 1990	36M/36F	Anthony
Athens control quail random-bred control w/ white eggshell mutation in gene pool, propagated by random-pair matings	Kept as closed flock since 1963	120M/120F	Burke
Louisiana random-bred quail unselected, random-bred population, some color mutations (tuxedo, redhead, white egg)	Kept at Louisiana State as closed flock for >20 years	60M/120F	Satterlee
Ohio R1 propagated using 36 pairs/generation	Derived from cross of Athens random-bred, Athens white egg, and Wisconsin stock; closed flock 38 generations	36M/36F	Nestor
UBC A random-bred flock	Derived from cross of UCD random-bred quail and quail stock from Korea; closed flock 70 generations	48M/96F	Cheng
UBC B exceptionally nervous random-bred; spade homozygotes have defective feathers	Acquired from U Alberta 1977, combined with UBC SP (spade mutation, affects feathers) by 1998	34M/48F	Cheng

Stock Name and Description	History of Origin	Number of Birds	Curator
UBC M rough-textured homozygotes have feathers that appear matted and rough; females produce fewer viable embryos (RT mutation not yet reported in literature); have extended brown allele; slightly heavier than UBC A	UBC M from commercial (Marsh Farms) strain in 1975; combined with UBC RT in 1989	24M/48F	Cheng
UBC N very docile random-bred	Acquired from U Nagoya (Japan), 1988	24M/48F	Cheng
UBC NC random-bred, sensitive to changes in photoperiod	Acquired from NCSU random-bred quail, 1990	34M/48F	Cheng
UBC S	Acquired from U Saskatchewan, 1983	24M/48F	Cheng
UBC WILD	Foundation stock was 12 feral birds caught in Hawaii, 1985	48M/96F	Cheng
UCD Random-bred quail wild-type feather color pattern, unselected, randomly grouped in colony cages (2M/4F); reproduce every 6 to 8 months	Derived from stock imported from Japan and Taiwan (1950s and 1972)	200+	Wilson
U Maryland random-bred quail	Acquired from U Wisconsin/US Department of Agriculture, 1970s; kept as closed flock for ~20 years	100	Ottinger
UNL Wild-type *Coturnix*	Acquired from U Georgia-Athens	30-60 pairs	Beck
Purdue *Coturnix* KGB – selected behavioral trait selected 18 generations for nonaggressive behavior, starting 1988	Derived from Athens control quail (U Georgia-Athens)	10,000 birds	Muir
Arkansas H10 – selected growth trait selected 18 generations for high 10-day body weight	Derived from Arkansas RBC	36M/36F	Anthony
Arkansas H17 – selected growth trait selected 18 generations for high 17-day body weight	Derived from Arkansas RBC	36M/36F	Anthony
Arkansas H28 – selected growth trait selected 18 generations for high 28-day body weight	Derived from Arkansas RBC	36M/36F	Anthony
Arkansas H40 –selected growth trait selected 18 generations for high 40-day body weight	Derived from Arkansas RBC	36M/36F	Anthony
Arkansas HL – selected growth trait selected for high early body weight gain (10-17 days), low late body weight gain (17-28 days)		36M/36F	Anthony
Arkansas LH -selected growth trait selected for low early body weight gain (10-17 days), high late body weight gain (17-28 days)		36M/36F	Anthony
Athens 52 high body weight selected	Derived from cross Athens 51 and 53 (both selected 38 generations for high 4-week body weight)	36M/36F	Burke

Stock Name and Description	History of Origin	Number of Birds	Curator
Athens 54 low body weight selected	Derived from cross of two stocks, both selected 38 generations for low 4-week body weight	36M/36F	Burke
Athens 56 – selected growth trait Intermediate body weight stock, cross of long-term selected high and low body weight stocks	Derived from cross of Athens P-, T-, and S- lines	36M/36F	Burke
Athens P-line selected 100 generations for high 4-week body weight, 28% protein diet; at 70 generations, adult size was >150% above that of controlled standard population	Derived from Athens control quail	60M/60F	Burke
Athens T-line selected 100 generations for high 4-week body weight, low-protein, thioruacil stress diet; resists growth depression on diets with up to 0.2% thiouracil	Derived from Athens control quail	60M/60F	Burke
Ohio HW inbreeding coefficient (F) = 0.417; selected 30 generations generations for increased 4-week body weight	Derived from Ohio R1	48M/48F	Nestor
Ohio HW-HP inbreeding coefficient (F)=0.375; selected for male increased 4-week body weight, for female increased plasma phosphorus (indicator of yolk precursors)	Derived from Ohio HW	36M/36F	Nestor
Ohio HW-LP inbreeding coefficient (F)=0.357; selected for male increased 4-week body weight, for female decreased plasma phosphorus 2 weeks after start of lay	Derived from Ohio HW	36M/36F	Nestor
Ohio LW inbreeding coefficient (F)=0.357; selected 30 generations for decreased 4-week body weight	Derived from Ohio R1	48M/48F	Nestor
UBC G-QM selected for average female body weight 280g at 6 weeks	UBC G derived from a commercial strain (Marsh Farms) combined with UBC QM in 1992	24M/48F	Cheng
UBC QF selected for average female body weight 280g at 6 weeks	Acquired from Deschambault in 1990	48M/96F	Cheng
UBC QM selected for average male body weight 280g at 6 weeks	Acquired from Deschambault in 1990, combined with UBC G in 1992	see UBC G-QM	Cheng
Bobwhite			
NIU bobwhites, blood type–gene pool various erythrocyte alloantigens in quail	Acquired from Mississippi State U in 1992	50+	Briles

a) Adapted from data in Pisenti et al. (1999).

Table 4-2. Body Weight and Reproductive Parameters of Egg-Producing and Meat Production Strains of Japanese Quail

Parameter	UBC Wild Type	Northwest Game Birds	UBC-QO	Quail Internt Georgia, USA	Texas A&M
Body weight (g) Male Female	 144 170		 232 297	 218 243	 312 331
Age at sexual maturity (days)	32 28-35		63 49-70	42	53 49-56
Number eggs/year	288	300	183	230	234
Number eggs/hen/day	0.79	0.82	0.5		0.64
Percent Fertility	87		90		
Percent Hatchability	90		85		

Reproduction is greatly affected by inbreeding in Japanese quail, and mating systems need to be selected to avoid pairing of closely related individuals. Sittmann et al. (1966) demonstrated a 66% decrease in hatch from one generation of full-sibling mating and nearly complete loss of hatch by the third generation. Fertility and hatchability decline by about 1% for each 1% additional inbreeding. Brooder mortality also increases substantially with each increase in inbreeding. Bateson (1983) demonstrated a mating preference for unfamiliar first cousins among Japanese quail; therefore, single-enclosure mating systems may be less successful than the more controlled mating systems in preventing inbreeding. To prevent inbreeding depression in resource stocks bred in single-enclosure systems where all males have access to all females, a minimum of 100 birds are needed. Japanese quail stocks are often maintained by random pairing or grouping in cages, in which case 25 pairs but usually more than 150 birds, are needed to keep inbreeding at a minimum (Pisenti et al. 1999). Typically, random-bred stocks are kept as closed populations; however, new bloodlines are often introduced to the flock if inbreeding depression is observed.

Japanese quail lay large eggs relative to their body size–7% to 8% of their body weight–with large yolk volume, providing a relatively large potential depot for xenobiotic transfer from hen to developing embryo. As is typical for nidifugous young, a reserve of yolk is gradually discharged into the blood plasma for several days post-hatch. Most strains of Japanese quail produce colored, often mottled eggshells that can make detection of cracks difficult. However, some strains produce light-colored or white shells (Poole 1964). Incubating eggs require a temperature of 37.5±0.3ºC and relative humidity (RH) of at least 60%. Egg incubations systems are available to automatically rotate the eggs every 2 to 4 h. Pedigree baskets are routinely used to track parentage of the young at hatch. Fertility of eggs stored at 14±0.3ºC and 70% RH is high for the first 7 days, after which it falls off dramatically. Rapid decline in hatchability occurs if the eggs are held longer than 7 days. Storage can be extended to 10 to 14 days, if the

eggs are covered in plastic to prevent desiccation (NRC 1969; Ottinger and Rattner 1999). The incubation period for Japanese quail is 14 to 18 days depending on strain. Humidity requirements during hatch are higher (70% RH minimum), and eggs can be sprayed with distilled water to help prevent embryonic membranes from drying after pipping (Olsen 1992; Ottinger and Rattner 1999). Hatchlings can be sexed by cloacal examination (Homma et al. 1966).

Japanese quail respond to sudden disturbance by flushing, and injury can result during capture, if the bird strikes against the ceiling of the cage. Cage height is therefore kept at about 25.5 cm to reduce the potential for injury. Some species, such as the feral Hawaiian strain, are considered flighty, whereas larger strains appear to be more docile. In all, they are relatively easy to handle, although manual restraint can occasionally induce tonic immobility, an instinctive fear response characterized by motor inhibition and loss of righting response for a period of time (Satterlee et al. 1993). Disturbing laying hens may cause a pause in egg-laying. Moving layers to new caging may result in a cessation of egg production for 2 to 3 weeks (NRC 1969). Interruption of egg-laying will have to be considered in selection of endpoints and/or sampling schedule.

The threshold for reproductive system stimulation in Japanese quail is 11 h of light and 13 h of darkness per day (11L:13D). Maximal photoresponse is obtained at 14L:10D and maintained at maximum up to 18L:6D. However, captive quail populations often display marked photoperiodic drift and large variability in reproductive response. This appears to be a result of the increasing population of individuals in stocks of *Coturnix* that reach sexual maturity on shorter day lengths. Dobson et al. (1992) reported up to 10% of a population may breed on 8L:16D photoperiods if selective breeding against early photoresponses is not maintained. Others have reported even greater percentages of test populations that enter into reproduction when raised under the recommended photoperiod for gonadal quiescence in avian reproduction tests (OECD Guideline 206). For example, Yamamoto et al. (1996) found that 60% of the Japanese quail used for a reproductive toxicity study entered into reproduction when maintained under the recommended short day length of 7 h to 8 h of light. When the day length was shortened to 6 h of light, 20% of the birds became reproductive. Decreasing day length to 5 h of light reduced the incidence of reproduction to less than 5% of the population. However, the reduced day length (6L:18D) resulted in lower food intake, and thus, reduced uptake of the test substance (fenthion), which in turn resulted in a much reduced response to the chemical exposure than was previously observed in their laboratory. Some strains of Japanese quail have been developed that do not require photostimulation of reproduction (Dobson et al. 1992). Photoperiodic drift within a strain of quail could compromise attempts to measure reproductive endpoints associated with sexual maturation (gonadal development, cloacal gland size, time to first egg laid, level of circulating hormones). Therefore, careful consideration should be given to strain selection prior to use in avian reproduction tests.

Light intensity is also important in maintaining quail. Light intensity that is too high will encourage aggressive behavior. The quail should be exposed to about 10 lux artificial light, measured at the level of the feeder, which approximates the daylight visual spectrum to initiate and maintain reproduction. However, to reduce aggression or maintain birds in nonreproductive

condition, lower levels of light may need to be provided. To ensure a stimulatory photoperiod as well as a long feeding period to maximize consumption of the test substance, a photoperiod of 16 h or 17 h of light and 7 h or 8 h of dark is commonly used. If the breeding colony is maintained on this light cycle throughout the year, the hens may experience premature aging from the calcium drain associated with constant egg-laying (Ottinger and Rattner 1999).

Coturnix can be maintained from hatchling through reproduction on a diet containing 26% to 28% protein (Howes 1965) and an energy content of 2000 productive energy calories per kilogram. High-energy diets should be avoided, because they cause steatosis (Howes and Fitzgerald 1966). Supplementary calcium (3.0%) is required for egg-laying and should be supplied just prior to maturity (Nelson et al. 1964). Commercial game bird and turkey starter diets are available and are commonly used for Japanese quail production. Most commercial and custom diets are formulated with soy and corn, which contain phytoestrogens in widely varying amounts. The impact of soy and corn diet on endocrine disruptor testing has not been studied. However, commercial turkey starter amended with extract from forage plants containing elevated levels of phytoestrogens (biochenin A and genistein) was shown to cause a delay in the onset of egg production in California qual (*Lophortyx californicus*) and to reduce egg production overall in this species (Leopold et al. 1977). Unfortunately, only relative concentrations–high, medium, and low–of the amended phytoestrogens were reported. A later study by Lien et al. (1987) showed that bobwhite would have to consume in excess of 1 mg/bird/day of biochenin A to adversely impact reproduction.

Stainless steel construction is recommended for caging used in avian toxicity tests, although galvanized construction is acceptable. Quail cages and brooders are commercially available in galvanized steel from production factories. However, stainless steel caging must be custom-constructed. There is an increasing interest in cages constructed of metal wire coated with perflourocarbon plastics to provide thermoneutral surfaces, ease of cleaning, and reduced cost. Some also feel that the coatings reduce egg breakage and may provide a more comfortable flooring for the birds. However, use of plastic water providers and food containers may contain plasticizers that should be avoided in tests measuring endocrine endpoints. Wire pens with slanting floors and egg-catchers or other measures to prevent breakage of eggs are recommended for adults.

4.1.3 Strains

A review of available documentation on genetic stocks of the Japanese quail sponsored by the University of California Genetic Resources Conservation Program indicated that all the strains of Japanese quail in North America and the European community developed through selective breeding for food production and/or biological research are descended from *C. japonica* (NRC 1985; Pisenti et al. 1999). Small wild populations of *C. japonica* still are found in Japan, and a random-bred population of the wild type is maintained at the Quail Genetic Resource Centre at UBC. A strain originating from feral quail captured on the island of Hawaii also is maintained at the center.

Many specialized genetic stocks of morphological and physiological mutations have been developed by universities and government research organizations (Pisenti et al. 1999). Likewise, research institutions and some commercial suppliers have developed and continue to conserve through randomly breeding populations a number of strains in which individual birds have many traits in common (Table 4-1). These random-bred strains are the sources of test organisms for current avian endocrine and reproductive toxicity studies conducted with Japanese quail (Pisenti et al. 1999). In the United States and Japan, quail currently used for toxicity testing and endocrine studies are derived from strains selected for their egg-producing traits. European studies often make use of strains of Japanese quail that have been selected for meat production. These strains may differ not only in growth rate and body size, but also in carcass composition (Barbato et al. 1984), sexual maturation, and egg production (Table 4-2). Increased lipid deposition in the form of body fat and yolk material is often observed in meat production strains fed *ad libitum* (Reddy and Siegel 1976; Barbato et al. 1984). Increased food consumption and lipid deposition in yolk could result in greater exposure of heavier birds to test substance in the feed and *in ovo*. To determine whether there are significant differences in growth and reproductive characteristics for the various strains of Japanese quail currently in use, researchers and commercial quail facilities in the United States, Japan, and the European community were canvassed to obtain data on strain maturation rate, productivity, length of peak production period, eggshell properties, aggression between members of a pair, reliance on photostimulation to induce reproduction, and sexual aggressiveness. This information is reported in Table 4-2.

In general, the smaller, egg-producing strains appear to mature more rapidly, have higher productivity, and retain peak fertility and productivity for a longer period than the larger, meat-production strains. However, there is some evidence in the literature that strains selected for rapid growth have more ovarian follicles in rapid development during the egg-laying cycle than do random-bred lines (Ye et al. 1999). This apparent contradiction may be explained by the higher incidence of follicles lost into the body cavity, follicular atresion, and production of abnormal eggs in strains selected for increased growth rate (Bacon et al. 1973; Nestor et al. 1982). Egg production of Japanese quail lines selected for high body weight at 4 weeks of age declined over generations (Nestor et al. 1996). Notably, in a random-bred line maintained using a paired mating system, nine reproduction traits declined over 30 generations (Nestor 1977). Many of these traits are related in the Japanese quail; therefore, changes in a single trait over the generations could be correlated with other traits (Nestor et al. 1995). The declining traits were egg production, hatch of eggs set, egg weight, albumen weight, shell weight, yolk weight, body weight at end of lay, and change in body weight during laying, and percentage liver dry matter. Selection for high body weight also appears to reduce the minimal requirements for males to advance to sexual maturity (Anthony et al. 1993). Selection is also made for docility, both for its effects on poultry welfare and on egg quality (Jones 1996). These quail tend to have reduced corticosterone responses (Satterlee and Johnson 1988).

How the various types of strains differ in sensitivity to EDCs is not known and should be evaluated to determine whether the differences would affect interlaboratory comparisons and hazard evaluation. An option currently being considered in Japan to address this issue of comparable test birds is to restrict test populations to those arising from a specific

egg-production strain and source for all endocrine-disruptor tests (Ito, Yoshihiko, Research Institute for Animal Science in Biochemistry and Toxicology, Kanagawa, Japan, currently Chair of the Endocrine Disruptor Task Force for Japanese Environmental Protection Agency, personal communication, 2002). However, criteria for selecting a standard strain or standard type strains must be carefully established and should take into consideration limits on body size, eggshell quality, fertility, photoperiod drift, behavior, and EDC sensitivity.

4.2 Bobwhite (*Colinus virginianus*)

The northern bobwhite is a New World quail only distantly related to quail of Old World origins (Hoyo et al. 1994). As a native species of the United States adaptable to laboratory conditions, the northern bobwhite has been used extensively under EPA and American Society for Testing and Materials (ASTM) test guidelines as a representative species for toxicological evaluation of pesticides and other environmental contaminants. Although there are many natural strains of bobwhite, few domesticated strains have been intentionally developed. Guidelines specifically call for bobwhite used on test to be phenotypically indistinguishable from the wild type to retain capacity to represent wildlife responses to chemicals. The life stages of the bobwhite are significantly longer than those of the Japanese quail, particularly the period from growth to reproductive maturity.

4.2.1 Natural History

The bobwhite is distributed from southeastern Ontario (Canada) to Guatemala and in Cuba but reaches its highest density in the eastern United States and Mexico. The species has been widely transplanted from pen-raised and wild-trapped birds in the United States and is well established in areas beyond its natural range, with disjunct populations found in Washington, Oregon, and Idaho. Northern bobwhite has been introduced also to British Columbia (Canada), Puerto Rico, Hawaii, and New Zealand (Hoyo et al. 1994). It is a ground-dwelling species with forest and grassland affinities. It also has adapted to intensive agricultural areas in the United States and to xeric ecosystems of Mexico and Central America. Bobwhite nests in the spring with clutch sizes of 10 to 15 eggs. The incubation period is 23 days, and nest success is usually between 20% to 40%. After the breeding season, family coveys of about 12 in its southern range and 15 in northern United States are formed. Body size also increases from south to north in its native range with birds in Chiapas, Mexico, averaging 129 g, whereas those in the eastern United States weigh about 172 g. The northern bobwhite is a sedentary species with a home range of 10 ha to 31 ha. It is an opportunistic feeder, consuming mainly seeds and invertebrates.

4.2.2 Availability, Culture, Handling

Numerous commercial and game farm sources of northern bobwhite are available. Most sources supply phenotypical wild-type birds for hunting and dog training. These or similar wild-type strains are the stocks also used or maintained by testing and research laboratories. Bobwhite husbandry is similar to that of the Japanese quail. Methods of incubation, brooding, maintenance of juveniles, and breeder support are generally the same as for the smaller Japanese quail. However, space requirements are greater, 400 to 900 cm^2 per adult bobwhite (Olsen

1993), and humidity requirement during incubation is higher (85% RH) for bobwhite (Ottinger and Rattner 1999). Similar to *Coturnix*, the northern bobwhite has a low photoperiodic threshold (10L:14D) for stimulation of the reproductive system (Kirkpatrick 1955), and also lacks the photorefractoriness of most other birds.

All life phases of the bobwhite are longer than those of Japanese quail. The incubation period is 23 to 24 days, development of sexually distinguishable plumage occurs at about 12 weeks of age, and sexual maturity is reached at 24 weeks of age (Ballard et al. 1994). Peak production is reached at about 6 weeks after the onset of lay. The northern bobwhite is bred to retain its flightiness, and excessive handling may result in mortality. Inbreeding depression will occur in bobwhite stocks under 200 pairs. Therefore, unrelated breeder stock is introduced into the resource stocks of bobwhite at least every 3 years to reduce inbreeding (*International Hatchery Practice* 1989).

4.2.3 Strains

There are 22 recognized subspecies of bobwhite (Hoyo et al. 1994). Five of the subspecies, *C. v. marilandicus, C. v. virginianus, C. v. floridanus, C. v. texanus*, and *C. v. taylori*, are native to the United States (Rosene 1969). The northern bobwhite is commonly bred commercially or by hobbyists as wild stock. Very little deliberate selective breeding of northern bobwhite has been practiced, with the exception of the bobwhite blood-type variants maintained at Northern Illinois University. However, a few strains, such as Eastern, for example, are selected for body weight for meat production. They are phenotypically similar to the wild type except for body weight and are generally not used in avian toxicity tests. However, because most suppliers raise bobwhite in game farm situations, there is some use of these meat strains by commercial testing laboratories when production of eggs and juveniles has decreased in winter. These larger strains are usually more docile and lay fewer eggs (*International Hatchery Practice* 1989).

4.3 Strengths and Weaknesses

Table 4-3 summarizes major strengths and weaknesses of the two species for two-generation tests. The major advantages of using the Japanese quail over bobwhite in a two-generation test guideline with endocrine endpoints are related to the very rapid incubation and maturation (Table 4-2) and high rate of egg production of the Japanese quail. These traits allow for the completion of a multigenerational test within a relatively short time, about 30 weeks, compared with 70 weeks for a bobwhite test. Further, they allow a large number of eggs per hen from which to sample for egg quality and chemical residue analyses. The cloacal gland of the Japanese quail provides an indirect measure of gonadal development, indicating reproductive fitness, and sexual maturation of the males. Because of the extensive use of *Coturnix* in biochemical and steroid research, endocrine and behavioral patterns in this species are well characterized (Hutchinson et al. 2000; Ottinger and Brinkley 1978, 1979a, 1979b). Spermatogenesis is most fully characterized in the Japanese quail (Lin and Jones 1992), which provides the ability to quantify histopathological evaluations of testicular function and to determine length of exposure required before spermatogonial damage could be detected

extragonadally (Section 5.1.1). No information is available on the seminiferous cycles of bobwhite.

Major disadvantages associated with the use of the Japanese quail are related to its extensive history of domestication. Stock populations are very sensitive to inbreeding and loss of fertility. Consequently, heed must be given to appropriate mating protocols in source flocks and to the parental and offspring pairing in a multigenerational test protocol. Also, many strains of Japanese quail are being used around the world for reproduction testing. These strains differ in body size, maturation rates, egg production, and lipid deposition in yolk. The impact of different strain selection on the outcome of a two-generation study is not known.

In contrast, the northern bobwhite is far less domesticated and thus faces less inbreeding pressure and presumably is more representative of wildlife responses. However, the comparative sensitivity of the two species to environmental chemicals, particularly those with potential endocrine effects, is not known. A study by Solecki et al. (1996) compared the reproductive effects of an OP pesticide on Japanese quail with those published by Bennett et al. (1990), but most of the reproductive impacts observed could be attributed to parental toxicity in both studies. Although no sensitivity comparisons in chronic reproductive toxicity tests have been conducted, comparison of the relative sensitivity of the Japanese quail and bobwhite to acute dietary exposure (Romijin et al. 1995) and to acute oral dose with pesticides (Romijin et al. 1995; Baril et al. 1994) have been made using data from the open literature and from studies in support of registration of pesticides. Both studies showed that the northern bobwhite tended to be more sensitive to more compounds than the Japanese quail, but the difference was not statistically significant. In the Romijin et al. study, the differences in sensitivity between the two quail species did not exceed a factor of 5. Sensitivity to some dietary mycotoxins was also greater in bobwhite chicks than in Japanese quail (Ruff et al. 1992). Aflatoxin, toxic metabolites produced by the mold, *Aspergillus flavus*, and T-2 toxin resulted in 40% and 23% mortality, respectively, in 2- and 3-week-old bobwhite. No mortality was observed in Japanese quail chicks fed the same contaminated diets. Body weight loss, feed conversion ratio, and mouth lesions were all less severe in Japanese quail (Ruff et al. 1992). Currently, a study directly comparing the relative reproductive sensitivity of the Japanese quail and northern bobwhite to known EDCs is in progress at the University of Maryland for the EPA. Until these results are available, existing data indicate that the sensitivity of the two species to environmental chemicals is comparable.

Table 4-3. Major Strengths and Weaknesses of Japanese Quail and Northern Bobwhite Related to Use in Avian Two-Generation Reproduction Toxicity Tests

Japanese Quail	Northern Bobwhite
ADVANTAGES	ADVANTAGES
• Endocrine, behavioral pattern characterized • Small bird; occupies 230 cm^2 per bird • Reach sexual maturity by 6 weeks • Prolific layer, close to 1 egg per day • Early maturity (36 days males, 42 days females) • Short incubation period (16 -17 days) • Males are aggressive breeders • Males maintain high fertility (90%) • Adapts well to breeder cages • Biological, physiological, biochemical data available • Produce a large egg (8% of body weight) • Naturally hardy in the laboratory • Highly photosensitive • Physiological aging is rapid and lifespan short • Dimorphism of plumage color and pattern makes it possible to identify sex at 3 weeks of age • Cloacal gland of the male may be used as measure of maturity • History of use in toxicity testing • Spermatogenesis is well characterized	• Less domesticated, wild type • Small bird; occupies 400-900 cm^2 per bird • Prolific layer (somewhat less than Japanese quail) • Males are aggressive breeders • Males maintain high fertility (95%) • Adapts well to laboratory • Produces a relatively large egg (8%-105% of body weight) • Hardy in the laboratory • Highly photosensitive • Populations not prone to photoperiodic drift. • Dimorphism of plumage color • History of use in toxicity testing • More yolk per egg (39.8% by weight) compared with Japanese quail (31.9%)
DISADVANTAGES	DISADVANTAGES
• Inbreeding not tolerated, (leads to impaired fertility) • Strains differ in body weight, maturation rate, egg production, lipid deposition in body and egg. • Populations can show marked photoperiodic drift with large variability in reproductive response • Food wastage making food consumption measurement difficult • Most strains have colored eggshells that are difficult to handle. Mottling makes detection of cracks in shell difficult (some white egg strains are available) • Less yolk per egg (31.9% by weight) compared with bobwhite (39.8%)	• Long incubation period • Long maturation period (24 months) • Food wastage making food consumption measurement difficult • Sex cannot be distinguished by plumage until 12 weeks of age • Lack cloacal gland • Spermatogenesis is not characterized

Also comparable are baseline ranges of reproductive parameters of the two quail species. Tables 4-4 and 4-5 show typical reproductive values for the Japanese quail and northern bobwhite compiled in a revised draft guideline for avian reproduction toxicity testing (OECD 2001). For additional comparison are shown the baseline values reported by Piccirillo and Orlando (1985) for evaluation of control data for one-generation reproduction studies using the bobwhite. Ranges for the Japanese quail will likely vary, depending on strain selection. To evaluate which of the quail species would be the better choice for increasing the power of an avian reproduction test to detect effects, by displaying less background variation in reproductive endpoints, Springer and Collins (1999) compared the results of two simulation studies. Using historical control data of reproductive parameters for Japanese quail (Baus et al. 1999) and northern bobwhite (Springer and Collins 1999), the two studies employed simulation methods to compare power of the respective test to detect a 20% change in the mean value of a treated group relative to controls. Power estimates for both species were high (>0.80) for ratio-type endpoints, such as eggs hatched /fertile eggs, but lower for count-type endpoints, such as eggs laid/hen. No similar power comparisons are available for endocrine endpoints.

Table 4-4. Typical Values for Reproductive Parameters in Japanese Quail and Northern Bobwhite

Parameter	Japanese Quail	Northern Bobwhite	
		OECD	*Piccirilo and Orlando 1981*
No. eggs laid/hen/day	0.66 to 0.89	0.40 to 0.81	0.6±0.004
Percentage cracked or broken eggs	0% to 10%	0% to 6%	8.2%±7.2%
Viability (percentage of fertile eggs that develop live embryos at 2/3 through incubation)	85% to 96%	72% to 98%	73.6%±19.9%
Hatchability (percentage of viable eggs that hatch)	70% to 80%	70% to 98%	82.0%±12.8%
Percentage hatchlings that survive to 14 days	85% to 97%	69% to 98%	76.3%±5.0%
Mean number 14-day-old survivors/hen/day	0.34 to 0.71	0.24 to 0.38	
Eggshell thickness (mm)	0.19 to 0.22	0.20 to 0.25	0.31±7.2%

Table 4-5. Comparison of Development Phases in Japanese Quail and the Northern Bobwhite[a]

Species	Candling for fertility and viability (days)	Incubation (days)	Hatch (days)	Dimorphism of Plumage (weeks)	Sexual Maturity (weeks)	Peak Egg Production after Onset of Lay
Japanese quail	8	15 to 16	17 to 18	3	6	3
Northern bobwhite	11	20 to 21	24 to 25	12	24	6

(a) Data adapted from OECD (2000).

5.0 EXPERIMENTAL DESIGN CONSIDERATIONS FOR TWO-GENERATION AVIAN TESTS

5.1 Exposure Duration

The exposure duration should be long enough for the test substance to reach equilibrium in the tissues and all possible effects on the reproductive processes to be expressed in the selected endpoints and provide exposure to all life stages.

5.1.1 Exposure of the Parental (P1) Generation (Pre- or Post-Egg-Laying)

Current EPA and OECD guidelines were originally designed to detect reproductive deficits resulting from chronic exposure to bioaccumulating substances. Therefore, birds are exposed under these guidelines well in advance of egg-laying so that the compound reaches equilibrium in the tissues and presumably a maximum exposure level in eggs. Newer pesticides are much less persistent, and significant reproductive effects have been detected with treatment periods of only 1 to 3 weeks (Bennett and Bennett 1990; Bennett et al. 1990; Rattner et al. 1982; Stromborg 1981, 1986). This led several authors to review the statistical and physiological advantages and disadvantages of the pre-egg-laying and post-egg-laying exposure regimens for one-generation avian reproductive toxicity tests (e.g., Collins 1994; Mineau et al. 1994; Bennett et al. 1990; Bennett and Ganio 1991; David Farrar, personal communication to T. Maciorowski, D. Urban, D. McLane, and D. Balluff, Statistical aspects of the evaluation of avian reproductive effects: accomplishments and objectives, 1995) and for the design of multiple generation tests that would include endocrine endpoints (Hart et al. 1999; Baus et al. 1999; Springer and Collins 1999; Bennett et al. 2001). These authors pointed out that exposure following proven breeding would allow for the removal of incompatible or infertile birds prior to exposure, which would remove a nontreatment-related source of variation and could increase the power of the test. Pretreatment measurements could also be used as covariates in the statistical analysis, again potentially increasing the power of the statistical test.

Conversely, exposure before pairs are proven to be breeders risks a loss of replication and corresponding statistical power due to infertility or incompatibility. Using control data from a variety of historical data sets, several investigators (Baus et al. 1999; Springer and Collins 1999; David Farrar, personal communication to T. Maciorowski, D. Urban, D. McLane, and D. Balluff, Statistical aspects of the evaluation of avian reproductive effects: accomplishments and objectives, 1995) studied the power of the proposed (OECD 2000) design of the avian reproduction test through modeled simulations of reproductive effects. These investigators showed that use of pretreatment measures as covariates in simulation tests resulted an increase in the power of the test for number of eggs laid and the number of eggs incubated that hatched for both the Japanese quail and bobwhite (Springer and Collins 1999). Although the simulation studies also demonstrated that significantly increased power can be obtained for all count variables by replacing nonproducing pairs of bobwhite (Springer and Collins 1999), replacing pairs in tests using Japanese quail appeared to be ineffective (Baus et al. 1999) or only slightly effective (Springer and Collins 1999) in increasing the power of the test. These simulation comparisons, however, were based on study designs that included only post-egg-laying treatment.

No simulation studies have been conducted that provide information for selection of treatment period based on power comparisons of pre-egg-laying and post-initiation of egg-laying exposure scenarios. However, Collin (1994) showed that the power of the FIFRA avian reproduction test (EPA 1982) wherein treatment is started prior to egg-laying is low. He determined that to have 80% power to detect a 20% decline in the number of hatchlings per eggs incubated required a sample size of 27 pens for hatchlings exposed to a test substance for 10 weeks prior to egg-laying and for 8 weeks after the start of egg-laying . To detect a reduction in the number of eggs laid in the pre-egg-laying exposure tests would require 74 pens. In contrast, Springer and Collins (1999) determined that only 16 pens would be required to detect a 20% reduction in these same parameters when treatment is started after the initiation of egg-laying and that only 12 pens would be required if the post-initiation of egg-laying test included a covariate. This comparison, however, is potentially confounded by differences in the assumptions, and the simulation and statistical methods between the studies. In particular, Collins (1994) used reproduction data from the entire study period rather than limiting it to the peak laying period of 5 to10 weeks used in the simulation reviewed by Springer and Collins (1999). Restricting the simulations to a period of high egg production when the number of hens in production and their rate of production are most stable appears to greatly enhance the statistical power of the test. Improved statistical power from this high-production period is probably the result of the increased median value of the endpoints (David Farrar, personal communication to T. Maciorowski, D. Urban, D. McLane, and D. Balluff, Statistical aspects of the evaluation of avian reproductive effects: accomplishments and objectives, 1995). Even so, the power values obtained in investigations that used only data from the peak production period in bobwhite varied greatly (Springer and Collins 1999).

When compared with the power increases obtained from screening out nonproductive pairs or using pretreatment data as covariates, using data from the period of high egg production appears to be more influential in increasing the power to detect effects in avian reproduction studies.

Springer and Collins (1999) reported a doubling of the power to detect effects in data from Weeks 3 to 8 after the onset of laying, as compared with Weeks 1 to 6 from onset of laying in bobwhite. Smaller increases of 24% to 32% were seen when nonlayers were removed or when a covariable was included in the analysis from simulations based on data from birds treated prior to egg-laying. Therefore, it is possible to increase the power of a test to detect effects in a study where the birds are exposed prior to maturation by analyzing data during peak egg production, that is, by excluding the low, variable egg production interval from statistical analysis. A proven-layer exposure also benefits from this approach, but in addition, has the ability to further improve the power to detect effects through screening out nonlayers and using pretreatment data as covariates.

Only one study was found in the literature that directly compared the results of a pre-egg and a post-egg-laying exposure regimen in reproductive toxicity studies (Bennett et al. 1990). Bobwhite in the pre-egg-laying test received methyl parathion, an OP insecticide, in their diet for a total of 25 weeks. Birds receiving dietary exposure of the insecticide after the onset of egg-laying were treated for 3 weeks during the peak egg-laying period. All dose-related reproductive effects that were found in the pre-egg-laying exposure study, including decreased food consumption and egg production, were also seen in the post-egg-laying study. Unfortunately, the advantage of using proven breeders to reduce variability in the measured endpoints was compromised by the abnormally large number of injured and nonproducing birds, a total of 40%, in the post-egg-laying study. The elevated number of injured birds was attributed to the higher rate of aggression in the post-egg-laying birds that was apparently a result of changing abruptly from the short to the long day-length light cycle. However, comparison of the two exposure regimens did demonstrate that initiating exposure to a test substance after the onset of laying significantly improved the dose-response relationship of certain variables by using pretreatment means as covariates for each hen. Variables that demonstrated an improved dose-response were those with lower within-pen variation than between pen variations such as eggshell quality (Hunt et al. 1977; Thompson et al. 1983).

From the available experimental and simulation data, it is clear that there is insufficient information to determine whether a post-initiation of egg-laying exposure will provide significant improvements over pre-egg-laying exposure in increasing the power of statistical tests to detect reproductive effects.

Besides the potential statistical advantages to be gained from the ability to remove nonproductive birds from the test before exposure begins and/or to use pretreatment data during egg-laying for each bird as a control, a proven breeder exposure can also provide information on the rapidity with which many reproductive effects are manifested in exposed birds. Information of this type is particularly useful in risk assessments of chemicals that undergo rapid degradation in the environment or have use patterns that preclude long-term exposure during the breeding season. Another advantage is the savings in time and cost that is attained with the reduction in exposure period from the 20 weeks of the prematuration exposure regimen to less than 10 weeks of the proven breeder regimen. However, it must be remembered that the reduced exposure period may also not be long enough for histological or biochemical lesions to develop.

There are potential reproductive effects, however, that cannot be detected in the P1 generation by exposing birds only after reproductive maturity. Of these effects, the age at which sexual maturation is attained is of particular importance to reproductive success and maintenance in wild bird populations. Altered rate of maturation is emerging as one of the more significant endpoints of endocrine disruption in birds (Yoshimura et al. 2000). In a study by Edens et al. (1976), age at sexual maturity was among the most sensitive measures of impaired reproduction in the female Japanese quail exposed to dietary lead from hatch through 12 weeks of age. Delayed sexual maturation was detected at dietary concentrations that caused no body weight loss or overt signs of toxicity. Onset of egg-laying was delayed by up to 2 weeks in treated birds. In turn, peak egg production was also delayed and in most of the treatment groups was not attained before termination of the test at 12 weeks of age. Such a delay in peak production of treated birds could confound a direct comparison not only of fecundity but also body weight, because female quail typically increase in body weight by 20% to 40% during sexual maturation. Exposure duration may need to be lengthened under these circumstances to characterize the delayed response in egg production. Conversely, chronic treatment with tamoxifen, an estrogen antagonist, resulted in precocious puberty and accelerated spermatogenesis in chickens (Rozenboim et al. 1986), similarly offering the potential to confound direct comparison with reproductive parameters in control and treated birds. A regression approach would capture the time-course of such events.

As pointed out in Bennett et al. (2001), the effects of delayed or accelerated maturation do not determine the ability of the F1 generation to reproduce successfully, because they are not transgenerational effects. Maturation information could be better attained in separate tests. Bennet et al. (2001) also noted that it is possible to obtain data to evaluate the effects of pre-egg-laying exposure from the F2 chicks, assuming the F1 chicks are treated prior to sexual maturation. Another alternative would be to add relatively inexpensive sexual maturation endpoints, such as cloacal gland size/function and day of first egg, to the avian one-generation test. The additional observations would also provide information on exposure duration to aid in identifying a delayed peak egg production period. However, if Japanese quail are used in the one-generation tests, strain selection becomes an important factor in assuring the pre-egg-laying exposure is adequate. As noted earlier, many strains of *Coturnix* exhibit photoperiod drift, resulting in the maturation of significant proportions of the population under regimens of <8 h light. Yamamoto et al. (1996) found that the day length had to be reduced severely to prevent the birds from entering into reproduction prior to the 10-week prereproduction period. The reduced day length resulted in reduced food consumption, and thus reduced uptake of the test substance in the birds.

Selection of an exposure regimen for the P1 generation of the avian two-generation test must also take into account the propensity of the test substance to bioaccumulate. For compounds that require a long time to reach equilibrium in tissues, an exposure period that begins prior to egg-laying may be necessary in order to reach a maximum exposure level in testicular tissue (Section 5.1.6) and in the egg. It is not clear, however, to what extent maternal transfer of contaminants to the egg is derived from stored lipid. Although residues of OCs in eggs and

tissues of females have been found to be highly correlated (Mineau 1982; Custer et al. 1990; Loncore and Stendell 1977; Bogan and Newton 1977), other studies indicated that dietary, not adipose lipid may be the primary source of fat-soluble contaminants in eggs of exposed birds (Norstrom et al. 1986; Roudybush et al. 1979). Until the source of contaminants in the egg is resolved under the conditions of the test, the potential for a delayed response in the F1 endpoints for bioaccumulating chemicals should be presumed and the exposure duration selected to accommodate the required build up in tissues.

5.1.2 Exposure of the Offspring (F1) of the Parents (No Exposure vs. Exposure from Hatch through Egg-Laying)

Bennett et al. (2001) reviewed the various potential exposure scenarios of the F1 generation. The two major exposure regimens involve 1) a worst case scenario, wherein the chicks are exposed from hatch through egg-laying to the same environmental concentrations as their parents, or 2) a nonexposure scenario wherein the chicks do not receive additional exposure other than *in ovo*. The former scenario is similar to the exposure regimen of classical mammalian reproductive toxicity studies (EPA 1982, 1985; OECD 1981) allowing observation of reproductive, developmental, and endocrine-mediated effects at all susceptible life stages, not just those expressed in the P1 generation or resulting from *in ovo* exposure. There is a real potential, however, that exposure to the test substance as the chicks mature will result in nonendocrine-related effects and potentially high juvenile mortality. This confounding influence of direct toxicity could mask the detection of endocrine-mediated effects. Limiting the F1 exposure to *in ovo* exposure (e.g., sexual differentiation) of the developing embryo would eliminate these potential interference and interpretative problems, and focus the test on the reproductive success of the F1 chicks. However, it is likely that for many chemicals, chicks will be exposed in the wild. Elimination of exposure during vulnerable growth and maturation stages may overlook impacts to reproductive success. Currently, there is no information available that would help to resolve the relative importance of treating the F1 chicks. It is important to the design of a two-generation reproduction test to determine whether endocrine disruption can be detected amid the responses induced by direct toxicity of compounds such as OP insecticides.

Because the potential exposure patterns will differ for different chemicals, effects from both the *in ovo* and continuous exposure regimens must be interpreted with caution, if the environmental exposures are likely to differ from the exposure regimen of the test. Continuous exposure is used for chemicals that degrade rapidly in the environment or in cases in which F1 chicks are not exposed to chemicals to which birds in the wild would be exposed throughout their life cycle.

5.1.3 Combined Exposure Scenarios for P1 and F1 Generations

The exposure scenarios for each generation should be combined in such a manner that the maximum number of reproductive processes and vulnerable life stages are exposed over the period of the study. In addition, the exposures should not mask endocrine-related effects or confound interpretation of results. The selection of exposures should also maximize time and cost-effectiveness of the test relative to the information obtained. From the discussions above, a list of desirable attributes were obtained and matched to the various exposure regimens (Table

5-1) regardless of the generation (P1, F1, or F2). In all exposure scenarios, the F2 generation is not exposed to the test substance. The combination of exposures that provides the most comprehensive number of the attributes with minimum disadvantages was determined (Table 5-1): post-egg-laying production exposure of the P1 generation; exposure of the F1 chicks through growth and egg-laying; no exposure of the F2 chicks. The resulting exposure combination provides a different exposure history for each generation such that the P1 generation receives a subchronic exposure during the reproductive adult stage; the F1 offspring are exposed continuously from *in ovo* embryogenesis through egg-laying; and the F2 generation is exposed *in ovo* only. By contrasting the P1 proven breeders and F1, the reproductive impact from full life cycle exposure from *in ovo* exposure by combined parental transfer and exposure of F1 birds through growth and breeding can be obtained. Contrasting the F1 with the F2 exposures allows evaluation of the contribution of *in ovo* exposure alone to the reproductive and endocrine-mediated effects of the test substance. Although effects on sexual maturation are included in the F1 exposure, it is not separated from effects induced *in ovo*. If this information were needed, a separate study could be performed, or maturation endpoints added to existing one-generation tests. A combination that employs the pre-egg-laying exposure of the P1generation would provide similar information as that previously described, but it would add direct maturation information. However, acquisition of these data would be at considerable time and labor cost and with potential loss of statistical power in ANOVA-based comparisons. Pre-egg-laying exposure could affect the F1 and consequently the F2 generation by influencing the transport of any of a number of essential maternal substances that are needed in the egg for normal development. In addition, if egg-laying were delayed or accelerated by prebreeding exposure, comparison of the periods of peak egg production between treated and control birds would have to be made with caution.

Table 5-1. Required Attributes of Parental and Offspring Exposure Regimens

	TREATMENT				
Parameter	**P1**		**F1**		**F2**
	Pre-egg	*Post-egg*	*None*	*Chicks*[a]	*None*
Increased power of test	----[b]	X	----	----	----
Detect altered maturation	X	----	----	X	----
Detect effects of *in ovo* exposure	----	----	X	Confound	X
Detect effects of chick exposure	Partial	----	----	X[c]	----
Worst-case environmental exposure	Partial	----	----	X	----

Exposure with advantages that cannot be replaced by other exposure scenario without consideration of cost	-----	X	----	X	(X)[(d)]
Relative time/cost savings	----	High	Moderate	-----	----
Exposure with advantages that cannot be replaced by other exposure scenario with consideration of cost	----	X	----	X	X

a) exposed from hatch through egg-laying.
b) ---- no effect.
c) Possible confounding of endocrine effects from direct toxicity.
d) Could be replaced by F1 no exposure.

5.1.4 Selection of Egg Cohort for F1 Breeding Pairs

The egg cohort from which the F1 breeding pairs will be obtained should be selected at a time during egg production when the full effect of the test substance on the production and quality of the eggs and the viability of the young have been attained. In tests where an exposure initiated after egg-laying has begun, effects in eggs may not appear for some time after the initiation of the exposure. For example, yolk is recruited by ovarian follicles up to 9 days before the egg is laid (Bacon et al. 1973) possibly delaying potential developmental effects of the test substance on embryos after the initiation of treatment. If the test substance has not reached equilibrium in the tissues, the period of maximum deposition of the chemical into eggs may be further delayed and maximum effects observed even later. As noted in Section 5.1.1, the source of chemical deposited in egg may be from the diet rather than fat tissue, in which case little extra delay would be observed. Another source of delayed response in birds exposed during breeding is the time required (17 – 21 days) for effects on early spermatogenic processes to be observed in eggs (see Section 5.1.6). Because of these and other potential delays in response after the onset of exposure, selection of the egg cohort for the next breeding population (F1) should be obtained as late as possible in the treatment period. Therefore, the last weekly egg batch at the end of the parental treatment period should be used for the F1 breeding pairs.

If treatment of the P1 birds results in a decrease in the number of eggs or in reduced hatchability, then additional eggs should be acquired by accumulating eggs from the last two batches of eggs. Eggs can be maintained for up to 14 days in cool storage. Care should be taken to reduce evaporation in the eggs during storage to extend their shelf life. Egg batches from which the F1 pairs will be obtained should be retained and composited before incubation and not incubated separately, such that two batches of chicks of different ages are produced. Maintaining pairs of

different ages would be difficult in practice and greatly add to the complexity of the experimental design.

5.1.5 Selection of P1 and F1 Birds for Pairing and Breeding

For the formation of the P1 breeding population, birds should be randomly paired and allocated to a treatment. More pairs than are required on test should be selected to ensure that there is the required number of breeding pairs in the control group at the end of the treatment period. This number can be estimated from the fertility rate of the source flock and the loss expected from aggression. If a proven breeder exposure regimen is used, pairs selected to be on test should have laid at least one fertilized egg.

Protocols for selecting chicks for the F1 breeding population must ensure that inbreeding is minimized and that the population is representative of the parental population. To minimize inbreeding, chicks are marked at hatch so that they can be traced to parental origin. When the gender of the chicks can be determined by plumage, the chicks should be randomly chosen and paired from the pool of available chicks for each P1 pair. The random pairing protocol will provide a mechanism for avoiding brother-sister pairing. If possible, the same number of chicks from each P1 pair both within treatments and between treatments will be represented in the F1 breeding population.

If exposure to the test substance has resulted in reduced chick production or an altered ratio of males and females, Bennett et al. (2001) suggested that it is permissible, within reason, to randomly select chicks from other P1 pairs within the same treatment to fill out the required number of pairs per group for the F1 breeding population. This unequal selection of the F1 chick population must be done with caution, because it could result in a cohort of birds less sensitive to the chemical than the average of the P1 generation such that subsequent generations show a reduced response. It may be possible to avoid back-filling the groups by accumulating eggs from the last 14 days of the treatment period rather than the last 7 days.

5.1.6 Comparison of One-Generation and Two-Generation Exposure Regimens

Originally designed to detect reproductive failure in birds exposed to bioaccumulating chemicals, the one-generation avian reproduction toxicity studies have extensive exposure periods. In the one-generation tests in current use (ASTM Method E1062; OECD Guideline 206; OPPT Guideline 850.2300), the parental birds are exposed to the test substance for 8 to 12 weeks prior to egg-laying. After egg-laying begins, the birds receive an additional 8 to 10 weeks of exposure. In the ASTM procedure, the duration of exposure after egg-laying has begun can be reduced to the equivalent of two clutches of eggs. Thus, the treatment period continues until the controls produce 25 eggs.

OECD (2001) proposed a two-generation avian reproduction toxicity test guideline; however, consensus on the appropriate exposure regimen has not been reached. Several combinations of exposure scenarios have been explored by the OECD Expert Group on Assessment of Endocrine

Disrupting Effects in Birds for the Endocrine Disruptor Testing and Assessment Task Force. In one scenario, the parental birds are exposed prior to sexual maturation and for 8 weeks after the beginning of egg-laying, much like in the exposure regimen for the one-generation studies. The F1 generation is either not exposed or exposed during all or certain stages of the birds' life cycle. The second P1 exposure scenario was proposed to address the more contemporary, short-lived compounds and to take advantage of enhanced statistical power from using proven breeders (Section 5.1.1). In this exposure regimen, the test substance is not administered until the females are proven egg-layers. Specifically, to be allocated to the test, a pair must have produced at least one egg during the last week prior to the start of a pretreatment period. Treatment begins 2 weeks after the beginning of the pretreatment period to coincide with the peak period of egg production in the Japanese quail. Birds are exposed to the test substance for 6 weeks. The various options for the F1 exposures are the same as for those that could be combined with the first P1 exposure regimen.

Because the exposure is relatively short, it is critical that the 6-week exposure will allow the detection of effects on germ cells to be observed. For female Japanese quail, depending on strain and age of the birds, 4 to 9 days are required for the follicles to recruit yolk (Bacon and Koontz 1971; Bacon et al. 1973; number of days may differ for other birds–see p. 54 this report and Bacon et al. 1973), and 24 h are needed for fertilization, formation of the shelled egg and oviposition (Johnson 2000). Assuming the test substance rapidly reaches steady state in adipose tissue and that the source of chemical is the adipose tissue rather than the diet (Section 5.1.1), the potential delay in effects would be at least 1 week. In contrast, some effects could not be observed for 3 weeks (half the exposure period) in male *Coturnix*.

To be able to detect any adverse effect on the earliest stages of sperm development, spermatogonial stem cells, would require a minimum exposure period of 17 to 21 days. Spermatogenesis, the time between the first division of the spermatogonia and the freeing of sperm by the Sertoli cell into the lumen of the seminiferous tubule (spermiation), occurs in 12.8 days in *Coturnix* (Lin and Jones 1992). During this time, germ cells within the seminiferous epithelium become committed to divide synchronously in stages of development and occupy a defined area within the tubule (Kirby and Froman 2000). For Japanese quail, the duration of one complete cycle of these stages is 2.69 days. Thus, sperm cells released from the germinal epithelium arise from spermatagonia that began differentiating 4.75 cycles earlier. Assuming that the test substance comes to equilibrium in the tissue within one cycle of the seminiferous epithelium, a minimum of about 6 cycles of the germinal epithelium, equal to 16 days, would be required to ensure that impact on early spermatogenic processes could be detected in lumenal sperm. An additional 24 h are needed for the sperm to move through the excurrent ducts of Japanese quail (Clulow and Jones 1988) to be collected in extragonadal (deferent duct or ejaculate) sperm samples. Longer exposure periods will be needed, if the test substance does not reach equilibrium in the tissue within the short 2.69-day cycle of the seminiferous epithelium. After copulation, the sperm are taken up by sperm storage tubules in the oviduct of the female, where they can be stored for 7 to 9 days (Schom and Abbot 1974). This could mean that an additional 7 to 9 days would be required before the first sperm exposed as spermatagonia would be available to fertilize eggs. However, last-male precedence phenomenon, by which the sperm

from the last male or last copulation will fertilize a disproportionate number of eggs as time passes, exists in birds and probably reduces the delay in sperm availability in the storage tubules to fewer than 4 days (Birkhead and Moller 1992). Therefore, in a 6-week exposure period, only during the final 3 weeks of this period will the full effects of the test substance on fertility, embryo viability, hatching success, and sperm quantity and quality be observed. No effective statistical method has been identified for analyzing such a delayed treatment effect.

Springer and Collins (1999) conducted simulation tests using an adaptation of the Roth step-down trend test incorporating covariates in the last 3 weeks, Weeks 8 through 10, of a standard bobwhite quail reproduction toxicity test. The use of these selected data resulted in a decline in the power of the test to detect two important variables, the number of chicks that hatched per number of eggs incubated and the number of hatchlings that survived 14 days. Little difference in power was observed for the other variables. Because *Coturnix* sustain peak production much longer than bobwhite, it may be that at the variability in production variables would be less in Japanese quail at the equivalent treatment period. If test substances required additional time to reach equilibrium than accounted for in the first cycle of the germinal epithelium, <3 days, then the 6-week exposure should be extended. Extending the exposure period from 6 to 8 weeks may be prudent both to better detect effects on early spermatogenesis and to avoid increased variability in preproduction in aging birds. The time course for spermatogenesis has not been characterized for bobwhite, and therefore, the necessary treatment period to detect effects in early spermatogenic processes is unknown.

In F1 exposure scenarios that extend through egg-laying, the statistical benefits from using pretreatment data as covariates and eliminating nonproductive pairs from test before treatment begins are not available. Therefore it is important to reduce variation within the test by comparing egg production and associated parameters during the more stable peak egg-laying period.

5.2 Route of Administration

Peroral routes of administration are the most common procedures used to introduce a chemical into test animals to assess its toxic properties (Tyler 1999). These routes are commonly used, because they can provide realistic uptake scenarios and relative ease in quantifying dose. Choosing the route of administration should be based on the type of exposure encountered by birds in the environment. Exposure via food or water is the most realistic of the routes, although continuous exposure at constant concentrations is unlikely, given the foraging ranges of most bird species. Compounds that are insoluble in water, or that are volatile or reactive could need to be administered orally by bolus. The strengths and weaknesses of these three peroral routes as they pertain to avian reproductive tests are discussed below.

5.2.1 Food

Oral exposure through contaminated food is the most common route used for chronically exposing birds to environmental toxicants. This route is used because administration of the test substance by diet with *ad libitum* access allows for a more natural exposure throughout the duration of the study and avoids the intermittent high body loading of bolus (also called gavage, or forced-feed) dosing. Although birds consume food throughout the day, a bolus-like exposure does occur with the typical gorge feeding at dawn and dusk (just after lights on and just before lights off in the laboratory setting) observed in many bird species; however, it is unlikely that the dietary bolus effect is as pronounced as that from gavaged doses.

A major disadvantage of dietary exposure compared with gavage dosing is that dose estimation (milligram per kilogram per day) is much less precise, because the test substance is not delivered directly into the digestive tract of the bird. The feeding habit of caged quail results in wastage of a great amount of food, which can increase feed requirements from 14-18 g/day to 30-35 g/day (NRC 1969), making accurate estimates of food consumption, and accordingly, the ingested dose difficult. Use of wire mesh over feed dispensers and other restrictions reduce billing out of feed, but any such control measures must not restrict *ad libitum* access to food. Also, some test substances can be unpalatable to the birds or cause anorexia (e.g., Stromborg 1986a, 1986b; Bennett and Bennett 1990) and consequently result in diet avoidance. In mammalian studies, bolus dosing would be employed to determine the toxicity and endocrine system impacts of such chemicals (Wilson and Hayes 1994). However, food avoidance or anorexia in birds is considered an important consequence of dietary exposure to chemicals in the environment (Bennett and Ganio 1991; Bennett et al. 2001) and bolus dosing is not often employed to otherwise determine impacts in avian reproductive tests. Therefore, measures of food consumption are essential to the interpretation of results from feeding studies.
Typically, dietary exposures are administered continuously at constant concentrations throughout an exposure period of several weeks. Continuous exposure to consistent concentrations for several weeks will almost always expose birds to a great deal more test substance than they would encounter in the wild environment, because they do not feed on 100% contaminated diet in the latter setting due to the large foraging ranges of most bird species and the relatively short environmental half-life of contemporary agrichemicals.. More realistic exposures could be achieved by reducing the concentration of chemicals in the diet based on natural degradation rates and the frequency of field applications. However, such dietary adjustments add complexity and cost to the exposure regimen.

Stability of the chemical in the feed must be verified to determine the frequency of diet preparation that will ensure that the birds receive a continuous exposure to the target concentration or one that is typical of attenuation rates in the environment. Most test guidelines require that the dietary concentrations not fall below 80% of the initial concentration (OPPT Guideline 850.2300; OECD Guideline 206). It is also essential to ensure that the test material is homogeneously distributed in the diet. Because some chemicals may bind to the feed, thus greatly reducing bioavailability of the substances, extraction tests should also be conducted to assure full exposure to the chemical. The chemical analyses required for these verification tests,

such as stability, homogeneous distribution, and bioavailability, typically exceed the analytical costs incurred in bolus treatments. However, the savings in labor from administering the chemical in the diet compared with the intensive labor required for daily bolus dosing (Section 5.1.3) usually mitigates the analytical costs of dietary treatment.

Incorporation of the test substance into commercially available game bird chow at different concentrations is relatively simple and should be accomplished whenever possible without the use of a carrier. Evaporative solvents or low-volatility solvents common to the food industry are used when needed to minimize confounding toxicity from carrier substances. However, plant-based carriers such as corn oil can contain endocrine-active compounds and should be tested for phytoestrogen content and either stripped of these materials, or an alternative noncontaminated carrier should be used. The stripping process should not add additional contaminants or alter the palatability of the feed. Endocrine-active compounds in the diet, whether feed is used as an exposure route or not, are also of concern. For example, soy and corn, which contain variable amounts of phytoestrogens, are the leading constituents of most commercial game bird and poultry diets. The effect on test results of diets containing natural estrogenic compounds is unknown, as is the effect of removing phytoestrogens from the diet. Diets amended with clover extract containing elevated levels of phytoestrogens delayed the onset of egg laying and reduced egg production in captive California quail (Leopold et al. 1975). In a comparative study of the relative potency of phytoestrogen and several synthetic estrogens, the synthetic estrogens–estriol, ß-estradiol-3-benzoate and diethylstilbestrol affected bobwhite reproduction at dietary concentrations of 100 µg/bird/day. The phytoestrogen biochenin A had no effect on reproduction at concentrations up to 1000 µg/bird/day (Lien et al. 1985).

Overall, administering the test substance in feed provides for a more environmentally realistic–if expected environmental concentration and chemical decomposition are taken into account–and cost-effective exposure than the bolus route (discussed in Section 5.2.3) and for a greater range of compounds than can be delivered in water (see Section 5.1.2).

5.2.2 Water

Water, like dietary treatment, is an ecologically relevant route of exposure for water- soluble chemicals, as long as adequate dose can be achieved. Because of the difficulty in maintaining uniform suspensions or emulsions of nonsoluble substances in water, this route is seldom used as the route of exposure for compounds with low water solubility. Concurrent contamination of the test substance with endocrine-active compounds or other contaminants in water is not as great a concern as it is in dietary exposures, because it is relatively easily purified prior to mixing. Dispersion is also more easily achieved for water-soluble test substances administered in drinking water. However, evaporation may result in concentration of the test substance and water dispensers that minimize evaporation should be used in place of trough feeders. The water dispenser should also provide a means of determining the amount of liquid consumed so that an accurate dose can be calculated. Water spillage is more serious than is feed spillage in determining dose, because recovery of spilled water is seldom feasible; therefore, careful monitoring of the water dispensers must be maintained throughout the dosing phase of a study.

The same precautions related to maintaining test concentrations of volatile chemicals in feed also pertain to those dispensed in water.

5.2.3 Bolus

Bolus is a peroral procedure, in which the test substance is fed to the bird through an intubation tube directly into the crop or proventriculus. Although usually delivered in liquid form by this method, material can also be delivered in gelatin capsules. It is used to simulate oral uptake when chemicals have physical and/or chemical properties that are not suitable for formulation in water or feed due to problems of stability or volatility, for example, or when the chemical is unpalatable. However, palatability and avoidance are important factors influencing reproduction in birds through decreased caloric intake as observed for some OPs (e.g. Bennett et al. 1990; Stromborg 1986a, 1986b; Bennett and Ganio 1991) and provides information that would be overlooked with bolus dosing. Although diet avoidance data are important, it must be remembered that contaminated feed can be replaced by other choices in food types and location in the field.

The greatest advantage of administering the test substance by bolus is that it can provide the most accurate dose estimate. The researcher directly administers a known amount of test substance to each bird based on the bird's body weight and does not have to account for spillage, evaporation, or consumption rates to calculate the dose. But because each bird must be weighed prior to each dosing event to calculate the dosing volume to be delivered, and each bird must be dosed by hand each day, this method is very labor-intensive over the course of a study. Assuming three treatment groups and a control group with 16 pairs of birds in each and a capture, weighing, dose calculation, and dose delivery time of only 5 min/bird, labor for dosing would exceed 10 h/day. Dosing a large number of animals rapidly also greatly increases the variability in bolus accuracy among technicians, and deaths from delivery of the dose to the trachea and lungs are not uncommon (Robens et al. 1994). Handling stress can also have impact to the health of the birds and has been shown to affect plasma levels of steroid and thyroid hormones (Williamson and Davison 1985a; Davison et al. 1985).

In contrast to the more gradual dosing by ingestion of a test compound in feed or water, administration of a daily dose of chemical in a bolus can alter absorption rates and saturate hepatic metabolic enzyme systems (Tyler 1999). Greater absorption rates result in higher peak plasma concentrations, and thus, lower test concentrations can be tolerated in bolus-dosed animals than in animals receiving feed or water treatment. Bolus dosing therefore can limit the achievable dose range. Also, saturation of hepatic enzymes from bolus doses can result in breakthrough of the parent chemical and higher concentrations (or lower concentrations of activated metabolites) in the systemic circulatory system than occur from the more natural dietary exposure, potentially affecting toxic and endocrine responses.

The timing and volume of bolus dosing are also important to the outcome of the test. Because gastric emptying time can affect the absorption and bioavailability of test substances, the dose is generally administered when little feed is present to interfere with absorption and that the various concentrations (milligram/milliliter) be delivered in a constant volume

(milliliter/kilogram body weight). The former requires awareness of the feeding pattern of the birds. Also, dosing on an empty gastrointestial tract is not similar to natural exposure, whereby food material already could be present to influence the availability and uptake of the test substance in the gut. In addition, regurgitation can result in significant (37% to 72%) loss of gavaged dose (Hart and Thompson 1995). Neither volume of administered dose up to 0.2% of body weight nor prior fasting affected the extent of regurgitation observed in starlings (*Sturnus vulgarus*) gavaged with chlorfenvinphos. Use of gelatin capsules did not reduce the regurgitation of the emetic pesticide (Hart 1993). However, gelatin capsules may allow administration of larger volumes of compounds without emetic properties. Splitting the dose volume between morning and evening periods may also aid in dose administration by capsule or liquid gavage. (. It should be noted that although vomiting has been reported for a wide range of avian species (Prys-Jones et al. 1973, Tomacc 1975, Zack and Falls 1976, Hart and Thompson 1995), the extent of regurgitation varies between the species and between chemicals tested.

When the test substance is not water-soluble, a corn oil vehicle is commonly used to dilute the test substance and administer the dose to the animal. However, it is increasingly recognized that use of this vehicle and other vegetable oils, mineral oil, and some emulsifying agents can have a profound effect on outcome of the test. In general, the uptake, peak plasma level, and toxicity of test compounds appear to be exacerbated by corn oil (e.g., Farooqui et al. 1995; Bull et al. 1986; Chieco et al. 1981). Use of corn oil increases lymph flow and the uptake of lipophilic compounds such as p,p´-DDT through the lymphatic system (Sieber 1976), thus potentially increasing the transfer of these compounds to the yolk and reducing the effect of hepatic portal first-pass circulation. In addition, corn oil contains endocrine-active substances that could confound reproductive and endocrine-related test results. Therefore, aqueous suspensions of less readily soluble test materials are often preferred over food oil vehicles.

There are also potential problems with intubation trauma of the tissues of the upper gastrointestinal tract that come in direct contact with the delivered bolus of chemical and of handling stress, particularly of laying hens, during the daily dosing regimen. Handling of laying hens can seriously reduce egg production.

Overall, the disadvantages of this dosing procedure–elevated cost, excessive handling, regurgitation of dose, potential for direct tissue injury, technician variability, loss of test subjects due to misplacement of dose, the influence of oil vehicles on response parameters, and limitations on dosage range–outweigh the advantage of accurate dose estimates. It should only be used when the stability or volatility of the test substance precludes using feed or water routes of exposure or when the test substance is not emetic, is administered in capsules, and sufficient experienced labor or reduced number of birds can be employed to minimize time and stress of dosing.

5.3 Dose Selection

Dose refers to the amount in milligrams of test substance administered to the treated animal. Dose can be administered as a constant daily dosage, amount per unit body weight, or as a constant dietary concentration. Constant dosage adjustments to account for different and changing food consumption rates in growing birds provide a means of comparing effects

between life stages and species of different body size. Dose adjustments are made, however, at the expense of worst-case exposures in young birds and the ability to compare results with environmental concentrations. Because both exposure methods are highly artificial compared with the dynamic changes in diet composition, contaminant loading, and consumption rates of wild birds during the reproductive season, the selection of exposure method should be determined by data needs of the risk assessment process that will be employed.

5.3.1 Dose Adjustment for Size

Selection of test concentrations for reproductive studies where the test substance is administered in feed is often based on providing a constant daily dose to the animal. An expected outcome of these tests is the determination of an NOAEL and/or a lowest observable adverse effect level (LOAEL), both reported in milligrams of test substance ingested per kilogram of body weight per day, because feed is not measured for individual birds, and body weight can change throughout the test; further, no reproduction study requires daily weighing of birds. These and other dosage values, such as toxicity values expressed as the amount of test substance administered per unit body weight to the animal, for example, provide a basis for comparing effects among individuals and species of widely varying body size. Also, during the rapid growth phase, young animals consume more food per body weight than adults, and thus consume more test substance per body weight per day than adults. Weight changes and energy requirements of reproductive adults also differ greatly from nonreproductive adult birds. Therefore, dietary concentrations of the test substance are adjusted to attain constant dosage among all animals within a treatment, regardless of size.

Maintenance of a constant daily dose ensures that the young are not exposed to concentrations above the maximum tolerated dose (MTD) and that the relative sensitivity of the life stages to the test substance can be evaluated. However, frequent diet adjustments would have to be made during the rapid growth phase to maintain a constant dosage throughout the treatment period. The frequency of dietary treatment changes should minimize the dosage differences between the beginning of an adjusted dose period and its termination. A weekly interval is commonly used in mammalian studies. However, for some portions of the growth curve, more frequent dose adjustments would be needed for the Japanese quail,. For example, a weekly dosing interval could result in the chicks' consuming a dose at the beginning of the period that is 43% greater than that at the end of the same dosage interval (Figure 5-1). Also, individual birds vary in their rate of food consumption and body weight at any given age, resulting in variable doses within treatments, even when the diet is adjusted. Adjusting dietary concentrations to compensate for changing food consumption rates requires weighing the birds to obtain weight-gain data on which to base the diet adjustments, and thereby introduces a great deal of handling stress into the study, as well. Labor and analytical costs to verify treatment concentrations are also increased by frequent diet concentration adjustments.

5.3.2 Dose Adjustment by Life Stage (Young vs. Adult)

The goal of avian reproductive studies has been to estimate risk to wildlife from exposure to expected field levels of a contaminant. Because chicks that will become the reproductive adults of the F1 generation would be exposed under worst-case scenarios to the same field levels of a contaminant as they would as adults, the effects of exposure during this life stage are important to the outcome of the test. That is, the results from tests designed around a constant dietary exposure are more directly comparable to environmental concentrations than those that adjust exposure by life stage.

There are disadvantages to this approach. The range of doses in reproduction studies are based on adult exposure to ensure maximum challenge to the reproductive parent; therefore, young birds consuming a proportionately greater amount of contaminated feed during their growth phase acquire a daily dose through their diet that will be considerably higher than that which they will ingest as adults. This can result in dose overlap, whereby the young are virtually in a different dose group than their parents for a period of time (Wilson and Hayes 1994). In Figure 5-1, growth and food consumption data from Marks (1991) were used to calculate the ingested dose at weekly intervals of birds that were consuming a diet containing 2-week-old Japanese quail and the 7-week old quail fed the same 100 ppm diet are significantly different (15.8 mg/kg and 9 mg/kg, respectively). If the dietary treatments were separated by a geometric factor of 0.6, the next highest treatment would be about 167 ppm. The ingested dose in a 7-week-old would be about 15 g or less than the amount ingested by the 2-week-old chick at the lower treatment level. Dose concentrations are usually based on either MTD of the reproductive adult or a multiple, such as five times, for example, the expected field concentration, to provide sufficient challenge to observe effects in reproduction studies. Therefore, by not adjusting down the dietary concentration to account for the higher rate of intake, the F1 chicks will receive higher dosages of chemical than reflected in the adult weight-based test concentrations. The greater ingested doses can be lethal to the F1 chicks and hinder evaluation of the effects of the test substance on the second generation. However, mortality of chicks due to direct acute toxicity may occur in the environment and could cause a valid reduction in recruitment for exposed populations.

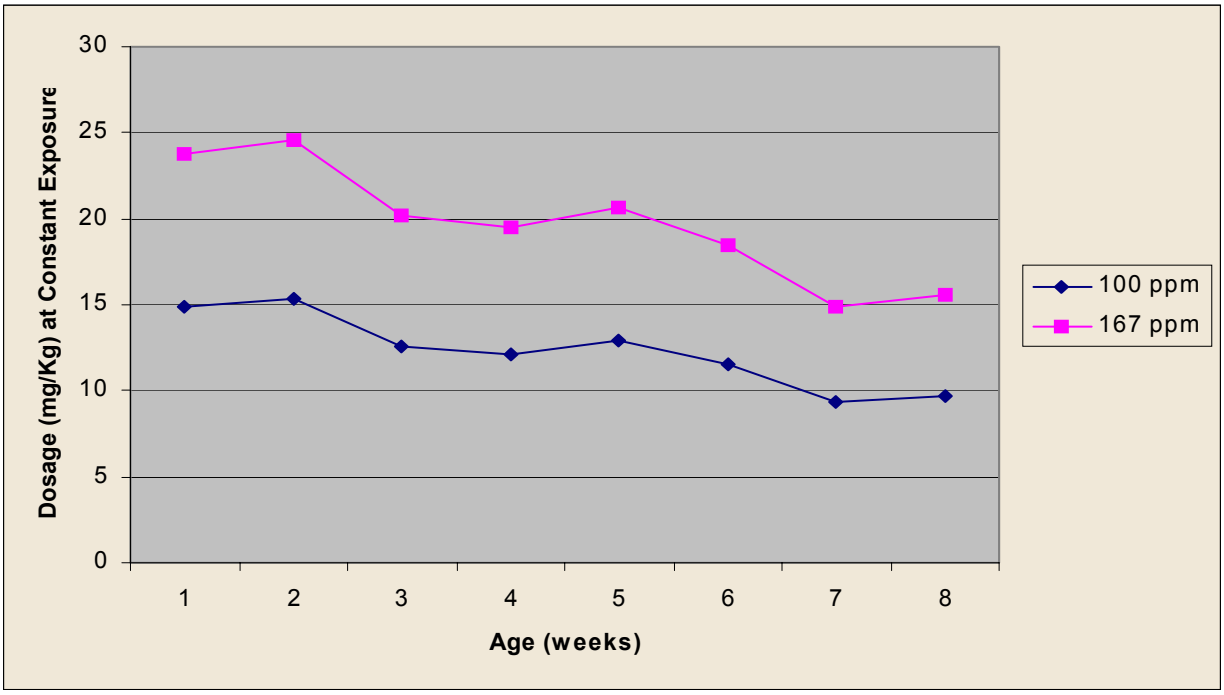

Figure 5-1. Calculated Dose at Weekly Intervals of Growing Japanese Quail Consuming a Diet Amended with 100 or 167 ppm of Test Substance (body weight and food consumption data from Marks [1991])

Comparison of chemical sensitivity among life stages is also complicated by the unequal daily doses among individuals and at different life stages. However, the logistics of periodically adjusting the dietary concentrations is eliminated by the consistent-exposure design, reducing both labor and cost of analytical verification of the different test concentrations. This is particularly important when using the Japanese quail as the test species, because numerous adjustments of test concentrations would be required to compensate for their rapid changes in body weight and to provide a reasonably constant daily dose at both the beginning of an adjusted

dose period and its termination. If an NOAEL or LOAEL is reported from data obtained under a constant exposure in feed, care should be taken that the value is represented as a dietary concentration in parts per million. Reporting values based on adult daily dosage (milligram/kilogram/day) is not representative of the doses experienced by the birds over the entire exposure period.

5.4 Statistical Considerations

Statistical approaches for interpreting avian reproductive toxicity tests have been reviewed by a number of authors. Bennett et al. (1990, 2001), Mineau et al. (1994), and Schlatterer et al. (1993) discussed the effects of mortalities and incompatibility of birds on sample size associated

with the timing and duration of treatment in reproductive studies using bobwhite and Japanese quail. A reduction in the sample size directly affects the power of hypothesis testing and the estimation of the NOAEL. Bennett and Ganio (EPA 1991) and MacLeod (1994) provided recommendations on determining which multiple comparison procedure should be used and how the testing should be carried out to estimate the NOAEL. Farrar (David Farrar, personal communication to T. Maciorowski, D. Urban, D. McLane, and D. Balluff, Statistical aspects of the evaluation of avian reproductive effects: accomplishments and objectives, 1995) and Collins (1994) compared the power of hypothesis-testing using specific endpoints measured in mallard ducks and bobwhite quail, and Springer and Collins (1999) evaluated the power of hypothesis-testing between bobwhite and Japanese quail. Chapman et al. (1996), Hart et al. (1999), and Crane and Newman (2000) suggested that the use of regression methods is a better approach to evaluating toxicity in avian reproduction tests, because they are less affected by the loss of replicates than are ANOVA methods and they estimate a dose-response curve that can be used to compare sensitivity both between endpoints and species. Baril et al. (1994) and Hart et al. (1999) discussed risk assessment using the estimated median lethal concentration (LC50) from the regression approach and alternative design strategies to reduce the number of birds on test. Recently, EPA evaluated and adopted a method based on dose-response curves as an alternative to the NOAEL approach to human health risk assessment (EPA 1995, 1999). This benchmark dose method[1] can evaluate both quantal and continuous data, and provide a risk reference dose that can be used like a NOAEL or LOAEL. Because this approach is based on regression methods, it incorporates information on the shape of the dose-response curve and can be used for both threshold and nonthreshold effects.

The objective of an avian two-generation reproductive and developmental toxicity test is to provide the most precise and accurate estimate of toxicity associated with endocrine disruption and reproductive fitness for an identified potential EDC. The results of the Tier 2 testing should be conclusive in documenting a discernible cause-and-effect relationship of chemical exposure to measurable manifestation in the test organisms. The test protocol will be designed to be capable of the following:

- to determine whether effects are a primary or secondary disturbance of endocrine function
- to establish exposure/concentrations/timing and effects relationships
- to be sensitive and specific
- to assess relevant endpoints
- to include a dose range for full characterization of effects (EDSTAC 1998).

Thus, the assay must be biologically sensitive, have minimal variability associated with dose exposure throughout the test duration, and have a statistically powerful inference. Biological sensitivity is a function of the choice of species tested, the relevance of the endpoints measured

[1]Benchmark dose (BMD) is defined as "a statistical lower confidence limit on the dose producing a predetermined level of change in an adverse response compared to [*sic*] a response in untreated animals" (EPA 1995). For example, a BMD_{10} would be the 95% lower confidence limit on a dose that produces a 10% increase in an adverse effect, such as the number of chicks with ovatestes.

to species productivity and survival, and the route, duration, and level of the chemical exposure. Design-associated variability in dose exposure is a function of exposure route and duration, chemical stability and purity within the testing environment, and the testing protocol. The power of a statistical inference is a function of the inherent variability in response; design-associated variability; the degrees of freedom and the source of variability for testing; and the estimation process and decision criteria.

Ideally, an experimental design incorporates randomness, independence, and replication (Cochran and Cox 1957). Randomness is used to remove noise, independence is used to extend the inferences made, and replication provides a measure of variability for testing (Chapman et al. 1996). Randomization of 1) experimental containers within a testing environment, 2) treatment application to experimental containers, and 3) assignment of organisms to experimental containers allows one to incorporate the variability associated with the environmental conditions, the containers, and the organism equally across all treatments. Thus, when one evaluates the difference between treatment means, the variability associated with experimental environment, experimental containers, and organisms being treated is removed and only the effect of the treatment remains.

Independence of treatment application and the creation of the treatment, and thus, the inference associated with the treatments under test, incorporates the variability associated with more than one individual, in more than one location, making and applying the same treatment. The random sample of organisms from a given population actually limits the inference to that population. However, one can evaluate the stability of the inherent variability of the population over time. An experimental unit is defined as the group of material or individuals to which a treatment is applied independently in a single trial of the experiment (Cochran and Cox 1957). Replication of experimental units for each treatment provides a measure of all the necessary sources of variability needed to extend the inference across time and space. A reduction in the sources of variability that are truly independent constrains the inference (Hurlbert 1984). Thus, if only one mix of each treatment is made and then divided among replicates, the source of variation associated with making the treatment is not included in the variability for testing, and the inference is limited. Some would say that this variability is nuisance noise, too small to be of concern. Therefore, if this source of variability is not included, it should at least be acknowledged. The variability among replicate experimental units could also include noise that was not randomized out due to a poor randomization or variable measurement error. These sources of variability can be reduced without loss to inference.

Statistical power is the probability of rejecting the null hypothesis of equal means when the alternative is true–that is, detecting a difference when there is a difference. Statistical power is a function of the variability among replicate experimental units within a treatment, the number of replicate experimental units, the size of the Type I error, and the percentage difference one wishes to detect. One can control the latter three components; however, the variability in response is inherent in the test organism. Thus, the choice of species to test and the relevant endpoints to measure should include a comparison of inherent variability or coefficients of variation (CVs), defined as standard deviation/mean x 100%. High CVs have low power for

detecting small-scale differences. For example, with 16 replicates per treatment, a CV of 50% would rarely detect differences less than 30% between the test and reference treatment response at a Type I error rate of $\alpha = 0.05$ (Figure 5-2). For a given CV, one can increase power by increasing the number of replicates. Test species and endpoints with the least inherent variability, by default, require the least replication for a given level of power and thus are more cost-effective.

Life-cycle studies provide a number of continuous and discrete random variables over the course of the exposure. Examples of continuous data include growth measured as size and weight, the gonadal somatic index, biochemical markers, and time to first laying. These data are analyzed using ANOVA and pair-wise comparison techniques to determine difference between treatments and controls, and regression or maximum likelihood techniques to estimate effective concentrations. Life-cycle studies also produce a large number of discrete data points, such as sex ratios, histopathology records, secondary sex characteristics, behavioral observations, survival, and fertilization or hatch success. These data can be analyzed by ANOVA if arcsine square root transformed, and by pair-wise comparison techniques, contingency table techniques to assess association, and regression or maximum likelihood techniques to estimate effective concentrations. Fisher's Exact Test is an example of a technique for comparing two sets of discrete quantal data.

Data collected by Schlatterer et al. (1993) for an interlaboratory comparison study on Japanese quail can be used to compare the CVs for a range of endpoints (Table 5-2). Five laboratories obtained 8- to 12-week-old birds from the same breeder and randomly assigned 12 pairs to the control diet. Fitness measures were then taken weekly for 6 weeks or from eggs laid during Weeks 5 and 6. The body weight of hatchlings, percentage of fertile eggs, and percentage hatch of fertile eggs had the smallest CVs for measurements with 10 observed means. Endpoints measured on eggs were not significantly different between Weeks 5 and 6. The CVs generally increased with dose; thus, these estimates are potentially quite low. In general, there are few data for which a power analysis can be conducted with great confidence. An increase in CV as a function of dose is dependent on the organism under test, the exposure chemical, and its toxicity to the endocrine system. A simulation study by Collins (1994) using control data from mallard ducks and bobwhite quail concluded that for birds caged 1:1, exposed over an 8-week period, 40 and 35 pens per treatment, respectively, were required to provide 80% power for detecting a 20% change in the affected variable. High CVs for these birds were confirmed by Farrar (David Farrar, personal communication to T. Maciorowski, D. Urban, D. McLane, and D. Balluff, Statistical aspects of the evaluation of avian reproductive effects: accomplishments and objectives, 1995).

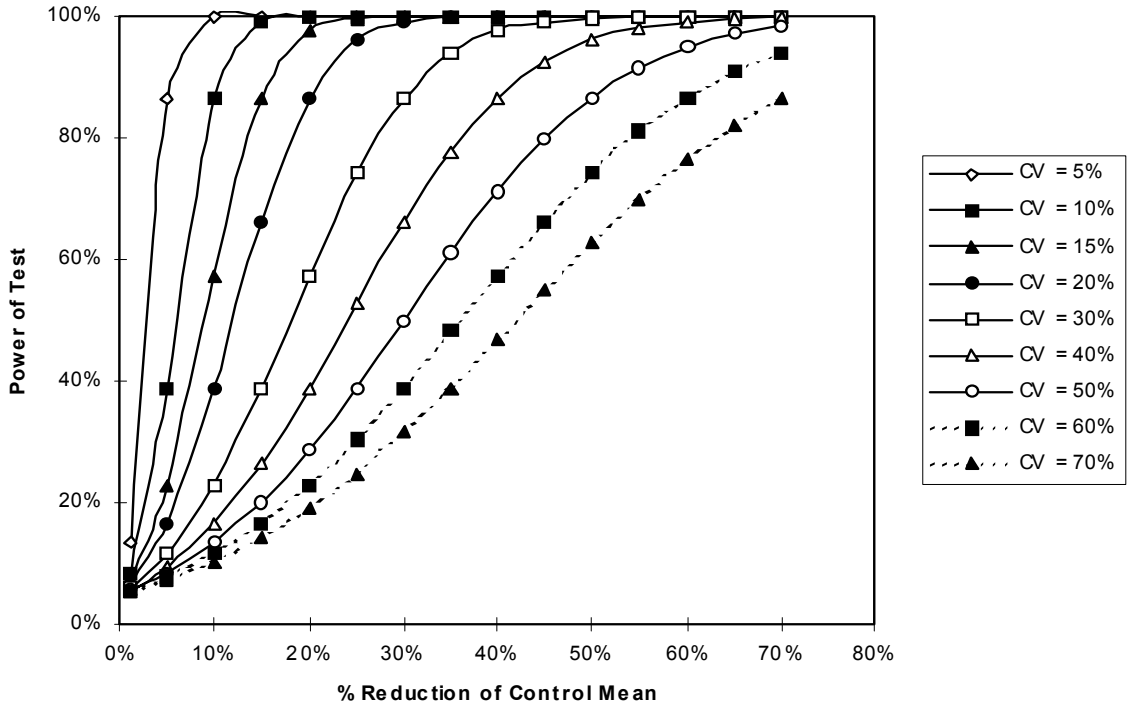

Figure 5-2. Power of a One-Sided Independent-Samples T-Test as a Function of the Percentage Reduction Detected Between the Test and Reference Means, with 16 Replicates per Treatment ($\alpha = 0.05$)

Table 5-2. Control Data Coefficient of Variation (CV%) for the Raw and Transformed Endpoints Measured from an Interlaboratory Comparison Study on Japanese Quail Exposed to Bis(tri—butyltin)oxide[a]

Variable	N	Raw Data (%)	Transformed Data (%)	Dose Effect Detected
Number eggs laid per week (intercept)	5	14	NA[b]	NS[c]
Mean egg weight per week (intercept)	5	3	NA	NS
Mean egg weight per week	2	6	NA	NS
Percentage cracked eggs	3	79	54	*[d]
Percentage fertile eggs	10	8	10	**[e]
Percentage hatch of fertile eggs	10	8	8	**
Percentage chicks dead in shell	4	22	12	**
Number hatched chicks	10	8	8	**
Body weight of hatchlings	10	6	3	NS
Number 14-day-olds	10	17	9	**
Weight of 14-day-olds	10	21	6	NS
Percentage surviving chicks hatched in Weeks 5 and 6	10	14	15	**

a) Table adapted from Schlatterer et al. (1993).
b) NA Not applicable.
c) NS Not specified.
d) * P< 0.05.
e) ** P < 0.01.

Springer and Collins (1999) stated that it is very difficult to make comparisons of power for hypothesis testing between bobwhite and Japanese quail, because the design of the studies have different starting times of exposure within the life cycle of the birds: that is, before maturation and after egg-laying, respectively. The effect of the different starting times of exposure is further confounded with the time required to reach chemical equilibrium in the bird's tissues. Thus, effects observed within Weeks 5 to 10 in bobwhite quail dosed at 3weeks may not be observed in Japanese quail dosed post egg-laying, until Week 12. Additionally, one must consider the decrease in variability, with increased power for testing, in the number of eggs laid as birds approach peak egg production (Springer and Collins 1999). Peak egg production generally occurs at Weeks 5 to 6 and Weeks 10 to 11 for Japanese quail and bobwhite, respectively.

5.4.1 Sample Size

5.4.1.1 Ensuring Adequate Number of Fertile Pairs. In the OECD draft guideline (OECD 1999), the initial test groups would consist of 20 replicate pens to increase the likelihood that at least 16 of them remain in each group at test termination. The highest concentration tested will be below levels shown to cause mortality or severe signs of parental toxicity from range-finding screening tests or from existing toxicological data, but will be of a level that is expected to reveal significant effects on reproductive and endocrine endpoints. Dosing of the P1 will commence at 4 weeks of age under one scenario, or after 2 weeks of egg-laying by proven breeders under a second scenario, and continue until test termination, depending on the resulting inherent variability observed in the preliminary tests. Both exposure scenarios have benefits, which are discussed in Bennett et al. (2001). Exposure before sexual maturation allows quantification of a delay in the onset of laying and gonadal development. Further, if the exposure chemical requires a period of time to build up in the tissues before the maximum exposure level in the eggs is reached, a delayed response in the F1 endpoints may be observed. In contrast, exposure before pairs are proven breeders risks a loss of replication–and statistical power–due to incompatibility or infertility. Indeed, Bennett et al. (1990) reported that 24 out of 60 pairs (40%) were removed from the test during the pre-exposure period due to mortality, injury, or failure to produce adequate numbers of eggs. Mineau et al. (1994), who reviewed 134 avian exposure studies, confirmed this high rate of infertility and mortality. For either exposure scenario, it is recommended that a population of birds not be used if more than 5% of either sex becomes debilitated in the 7-day period immediately prior to test initiation.

Hart et al. (1999) and Bennett et al. (2001) both reported that exposure following proven breeding allows the pretreatment measurements of fitness to be used as covariates in the statistical analysis. In some cases, the covariate data can increase the power of the statistical test. Removal of incompatible or infertile birds prior to exposure also removes nontreatment-related sources of variation, which again can increase the power of the test.

5.4.1.2 Ensuring Adequate Number of Offspring For Testing of Successive Generations. The variability of an endpoint increases with the number of phases of the reproductive process being tested. Thus, there is less power in detecting differences in 14-day-old survivors than for detecting differences in the number of eggs laid (Hart et al. 1999). Additionally, oral exposure could reduce food intake, thus affecting the exposure dose and resulting egg weights for several days of testing. Schlatterer et al. (1993) and Bennett et al. (1990) showed that a reduced food intake at higher doses was correlated also with greater impairment of fertility rate or a cessation of hatching for Japanese quail and bobwhite, respectively. For exposure scenarios in which treatment is initiated after a period of proven egg production, chicks raised for the F1 breeding population should be taken from egg batches produced at the end of the parental treatment period, thus allowing time for the full effect of the treatment to be established on the egg production and viability of young.

The F1 populations will be divided into groups of 20 replicate pens according to their parental test diet and will either receive no treatment or the same treatment as the parental diet. If

treatment groups experience a significant reduction in the number of F1 chicks produced, eggs produced over the last 10 to 14 days can be accumulated. Thus, all F1 birds will be the same age. For treatments with insufficient chicks for pairing, either back-filling, the effects of which are discussed in Section 5.1.5, would be employed, or the test continued with an unbalanced number of replicates, or the treatment discontinued. For any of these strategies, one must account for the objective of this experiment, which is to produce the worst-case scenario; however, the natural variation in the species to respond to the contaminant will by default produce more successful F1 pairs that are not as susceptible to the contaminant.

5.4.2 Hypothesis-Testing or Regression Analysis

There has been much debate over the use of the NOAEL in toxicity assessment and the associated risk analysis (Bennett et al. 2001; Crane and Newman 2000; Chapman et al. 1996). The debate stems from the perceived goal of the avian reproductive test: to detect effects on the reproduction of the test population at the lowest dietary concentrations that produce biologically significant effects (EPA 1991). The desire to detect effects implies a comparison of means. ANOVA methods are appropriate for comparing means, asking the question of whether the treatment means are statistically different from the control, such as in a screening test or a validation test. However, ANOVA methods are not appropriate when a precise and accurate estimate of toxicity and the pattern of response are required. There is also a false positive error rate in ANOVA, because the many parameters assessed are not all independent. Regression techniques provide an estimate of the level of effect as a function of exposure (nominal or actual concentration) and the functional relationship between dose and response. Further, by analyzing the different dose-response relationships, one can compare the sensitivity and potential thresholds of effect for different endpoints.

Although the NOAEL is used widely, it should not be relied on as the sole indicator of low toxicity (Crane and Newman 2000; Chapman et al. 1996; EPA 1991). The largest dose for which statistical differences have failed to be detected is a direct function of the power of the test: failure to reject the null hypothesis of no difference does not mean that there was no effect. For example, for certain endpoints with CVs greater than or equal to 40%, it is unlikely that reductions less than 30% will be detected with 16 replicates per treatment (Figure 5-2). It is also conceivable that short-term range-finding experiments will have difficulty in predicting the location of a NOAEL in a multigenerational test. It may also prove difficult to achieve effects bracketing the 50% response in the F1 population. However, effect concentration calculations are an appropriate alternative for estimating doses associated with low to medium toxicity. Care must be taken not to estimate an effects concentration that is more sensitive than the data and the experimental design will allow. Precision and accuracy of the effects concentration is a function of the spread between treatment concentrations and the number of concentrations tested (Chapman et al. 1996).

The design and analysis requirements for estimating the NOAEL differ from those for fitting a dose-response model (Chapman et al. 1996; Stephan and Rogers 1985). ANOVA methods require experimental unit replication and achieve greater power in testing as a function of the

number of replicates. As shown in Figure 5-2 and Table 5-2, the different endpoints would require different amounts of replication to achieve the same level of power. However, 16 replicates provide greater than 80% power for detecting less than a 20% change at $\alpha = 0.05$ from the control for most of the endpoints in Table 5-2, assuming the CVs do not increase with dose beyond 20%. Transformation of the data to satisfy homogeneity of variance is required for the parametric test and the regression approach. Estimation of the NOAEL does not require the assumption of a specific model, such as log-normal, and ANOVA methods, such as the t-test and Dunnett's test, are robust to non-normal errors (Scheffé 1959).

The design of a study intended for dose-response modeling does not require replication of the treatments (Snedicor and Cockran 1980). Each individual responses is assumed to be a random response from a normal population of responses for a given dose. The variance is assumed to be equal for each population. Replication of doses provides a test of equal variance and lack-of-fit (Draper and Smith 1981). Further, because of the unpredictable nature of survival, fertility, and compatibility of birds in the two-generation test and the large variability in specific endpoints, it is desirable to have some level of treatment replication to provide a more accurate estimate of the mean population response for a given dose. The number of replicates would depend on the maximum expected variability in response for each dose. The variability in response may be a function of the dose. In this case, either a weighted analysis should be conducted or a data transformation applied that satisfies the assumption of homogeneity of variance.

Benefits of the regression approach include 1) estimation of the pattern, or slope, of toxicity as a function of dose; 2) estimation of the distance between effect concentrations and environmental concentrations; 3) estimation of effective doses (ED*x*) and their associated confidence intervals for *x* equal to a low to medium effect; 4) estimation of ED*x* not limited to doses on test; 5) use of both measured and nominal concentrations; and 6) ability to compare dose-response curves across endpoints (Chapman et al. 1996; EPA 1991). The size of the resulting confidence intervals, indicating the precision of the estimated ED*x*, is a function of the inherent variability in the response, and the number and spacing of the concentrations tested. Guidelines often require five concentrations that are geometrically spaced and sublethal, plus a no-dose control. Thus, a range-finding test would be required to determine appropriate dietary concentrations.

Regression modeling is sufficiently flexible to handle a wide range of dose-response patterns, including nonmonotonic. If only one or two responses are not either 0% or 100% affected and at least one is greater than 50% affected, the Spearman-Karber nonparametric method can be used to estimate a median effective concentration (EC50). Finally, the regression approach can handle a wide range of responses, including continuous responses, counts, and quantal data, by re-expressing or transforming the data (e.g., log[y+c], [y+c]1/2), and probit, respectively).

Regression analysis on the weekly post-treatment responses also allows an evaluation of the resulting time-series and a potential redefinition of the effect of treatment (Hart et al. 1999). For example, a regression of the rate of egg production against time elapsed can be used to assess the shape of the response, the daily within-class variation in response, and the potential time-lag between exposure and response.

An alternative design that has been suggested for estimating a median lethal dose (LD50) and its associated confidence interval uses an up-and-down procedure. The intent of the design is to minimize the number of animals tested. A simulation experiment (Hart et al. 2001) concluded that the precision of the up-down estimate was not as good as that achieved from the regression analysis. However, the achieved precision from the up-down procedure may be acceptable. For steep dose-response relationships, the starting doses and step lengths did not seem to affect either the estimate or the precision. Often, the number of birds required was too low for an estimate of the slope of the response. For shallow slopes, the precision was much less than that achieved by regression, and the step length seemed to affect the outcome. It is not clear whether this type of design would be applicable to two-generation testing.

6.0 ASSAY ENDPOINTS: FITNESS END POINTS

Fitness endpoints are those that contribute to the normal functions of the animal, and thus relate to survival, growth, reproduction, and behavior of a test subject. Endpoints useful to assessing the reproductive and developmental toxicity of chemicals over two generations include those that provide information on general toxicity of the compound related to reproductive success, and those that measure the disruption of endocrine-mediated processes that can affect production. There are four life stages in precocial birds, such as the Japanese quail and northern bobwhite, during which critical endocrine-mediated processes occur. These potentially endocrine disruption-sensitive life stages are 1) the sexual maturation period of the P1 and F1 generations; 2) egg-laying of the P1 and F1 generations; 3) embryonic development (*in ovo*); and 4) early chick growth (F1 and F2). Table 6-1 lists the potential fitness endpoints that could be used in a two-generation reproduction test. In the table and in the discussion below, the endpoints are related to the life stage in which the endpoint could be measured. This table is adapted from a discussion paper on prevalidation of an avian two-generation toxicity test by Bennett et al. (2001) for the OECD Expert Group on Assessment of Endocrine Disrupting Effects in Birds. The table is modified to reflect those endpoint measures that tend to cluster or segregate into groups of highly related variables in avian reproduction studies (Mineau et al. 1994) and to include those recommended by EDSTAC (EPA 1998). These clustered variables reflect effects of the test substance on eggshell quality, developmental effects, and parental toxicity, and are so indicated on the table. Because an alternative to proscribing a fixed set of endpoints for all compounds tested in a test guideline, is to design a test with a core set of endpoints to which can be additional endpoints based on anticipated mechanism of action, the potential underlying estrogenic, androgenic and thyrogenic endocrine mechanisms are also provided in the table (after Bennett et al. 2001).

Table 6-1. Fitness Endpoints Specific to Endocrine Active Substances[a]

Fitness Endpoints	Critical Life Stages				Type of Endocrine Activity		
	Embryo genesis	Early develop-ment	Sexual maturation	Egg-laying	Estrogenic	Androgenic	Thyroido-genic
For F1 and F2 chicks							
number eggs laid per pair				yes	+(b)		
number fertile eggs per eggs laid PTOX[d]				yes	+/–(c)	+/--	
number cracked eggs at set and at 2 weeks ETOX[e]				yes			
number eggs hatched per eggs set DTOX[f]	yes			yes	+/--	+/--	
number chicks surviving to 7 and 14 days per eggs set and per eggs hatched DTOX		yes					
growth rate of chicks (weight at Days 1, 7, 14)		yes			+/--	+/--	+/--
eggshell strength or thickness ETOX				yes			
early and late viability per eggs set DTOX	yes				+/--	+/--	
sex ratio of chicks	yes		yes		+/--	+/--	
behavior at 14 days of age Visual cliff test Cold stress test Nest attentiveness	 yes yes yes				 – – –	 + + +	
For Parents							
body weight at start and end of treatment PTOX			yes	yes		+/--	+/--
food consumption weekly during treatment PTOX			yes	yes		+/--	+/--
male copulatory behavior			yes	yes			
signs of toxicity			yes	yes	–	+	
survival			yes	yes			

Fitness Endpoints	Critical Life Stages				Type of Endocrine Activity		
	Embryo genesis	Early development	Sexual maturation	Egg-laying	Estrogenic	Androgenic	Thyroido-genic
For F1 Juveniles to Adults							
age at onset of egg-laying			yes				+/--
food consumption weekly during treatment		yes	yes	yes		+/--	+/--
body weight weekly		yes	yes	yes		+/--	+/--

a) Adapted from Bennett et al. (2001).
b) + indicates positive activity (e.g., estrogenic, androgenic, thyroidogenic).
c) – indicates negative activity (e.g., antiestrogenic, antiandrogenic, antithyroidogenic).
a) PTOX indicates endpoints that segregate into a group that reflect effects on *parental* health and reproduction.
e) DTOX indicates endpoints that segregate into a group that reflect effects on *development.*
f) ETOX indicates endpoints that segregate into a group that reflect effects on *egg shell quality.*

6.1 Growth Rate, Food Consumption

Food consumption, maintenance of adult body weight, and growth of chicks are important indices of health status and reproductive fitness of each generation. Body weight is often the most sensitive measure of effect in animals exposed to xenobiotic chemicals and is analyzed either as change in body weight or as absolute body weight. Using rates of body weight change has the advantage of increasing the initial sensitivity of statistical analysis. The increase in sensitivity is a result of setting the initial weights as a zero value, thus reducing the amount of initial variability (Gad 2001). Body weight is also used to ensure that treatment and control groups contain animals of equivalent health status and body condition at the beginning of toxicity tests. Proper randomization of the animals accomplished when no group differs significantly in body weight from the mean body weight of the other groups, and all the animals on test are within two standard deviations of the overall mean body weight (Wilson and Hayes 1994).

Food consumption, by affecting body condition, can influence sexual maturation and the quality and number of eggs and subsequent hatchlings produced. In a statistical evaluation of 134 avian reproductive toxicity studies, Mineau et al. (1994) showed that the average adult body weight and food consumption during egg-laying correlated highly with the number of eggs laid. To produce eggs, quail must nearly double their daily food consumption (Case 1972). Thus, reduced caloric intake, resulting in poor body condition, as well as direct effects on reproductive processes, can result in reduced fecundity in birds (Mobarak 1990). Underfeeding has been shown to induce endocrine responses, including reduced levels of circulating hormones and gonadal weight (Mobarak 1990). Bennett and Ganio (1991) reviewed the relationship between food consumption and reproductive success in avian reproduction studies. Using pair-fed controls, some researchers demonstrated equivalent reductions in reproductive capacity in birds treated with OP pesticides (Stromborg 1986a, 1986b; Bennett and Bennett 1990). Other OP pesticides appear to induce reproductive deficits above those attributable to reduced caloric intake alone.

Growth rate and food consumption have a long history as endpoints for assessing the impact of endocrine agents in the poultry industry. Estrogenic compounds were used in the poultry industry for many years as a substitute for surgical caponization and were found to markedly increase body weight. Because estrogens are hyperlipidemic in avian species, much of the weight gain is in the form of abdominal and muscle fat (Snapir et al.1983). In support of this weight gain, food consumption is increased. In contrast, androgenic compounds appear to inhibit growth in birds prior to sexual maturity by terminating bone growth. However, androgens are anabolic after sexual maturity and epiphyseal closure (Weppleman 1984). Antiandrogenic compounds also have the potential to alter normal rates of growth and food consumption, as do thyroidogenic compounds. Regulation of the metabolic rate of poultry as a means to affect growth has involved various methods of controlling thyroid function, such as dietary supplements with thiouracil, for example (Moreng and Avens 1985; Marks 1992). These studies employed body weight or a body weight/food consumption index as an indicator of thyroidogenic or antithyroidogenic effects. Body weight measurements of the female and male, if cohabiting, should not be obtained during the egg-laying period. Fearfulness has been positively correlated to the numbers of abnormal, cracked, and body-checked eggs in poultry (Ouart and Adams 1982; Jones and Huges 1986; Mills et al. 1991).

Quail feeding behaviors result in a great deal of food scattering, even when screens and other devises are used to reduce wastage; therefore, food consumption data can be quite variable and should best be considered an estimate rather than a absolute value. However, food consumption data are essential in the interpretation of body weight changes to assess effects of treatment on eating and feed efficiency (feed consumed/grams weight gain), and to calculate consumed dosages (milligrams of test substance/kilograms body weight).

6.2 Measures of Reproductive Performance

6.2.1 Fecundity

As a measure of reproductive success, fecundity is widely used in oviparous animals to provide insight into potential effects of xenobiotics at the population level. In avian reproduction toxicity tests, fecundity is measured by the number of eggs laid per pen within treatment groups. It was highly correlated with adult body weight and food consumption in past avian reproductive toxicity studies (Mineau et al. 1994) and is affected by environmental conditions, age, and intraspecific genetic variation (McNabb et al. 1993). In females, the number of eggs laid is the difference between oocyte recruitment and atrecia (oocyte degeneration) in the ovarian hierarchy of oocyte maturation (Ryan 1981). In males, fecundity is function of semen quality and the number of sperm that have the potential to successfully complete all steps in the fertilization process: sperm movement, storage in the females sperm storage tubules, binding and penetrating the perivitelline layer, and fertilization (Donoghue 1999).

Indeterminate layers, such as Japanese quail and bobwhite, are used in reproductive studies, because they can provide a large number of eggs over a long laying period. However, using the total number of eggs produced per pen over the entire course of a test as an endpoint is problematic. Maximum egg production occurs about 3 or 6 weeks after the onset of laying for

the Japanese quail and northern bobwhite, respectively. Egg production prior to the peak laying period for these species is low, highly variable, and likely to result in a loss of statistical power when incorporated with data from the subsequent higher, more stable period of maximum egg production (Springer and Collins 1999). Issues of attaining chemical equilibrium in the tissues of the bird and delayed toxic response after one or two gamete cycles, also impose restrictions on using early eggs in a single number endpoint. Similarly, fecundity data collected for 10 weeks after the onset of lay will increase in variability as the aging breeders terminate egg-laying. A compromise between having a large sample and reducing intratreatment variability is to collect for fecundity measurement only those eggs produced during peak production and for only as long as maximum production is maintained. This alternative approach was suggested in the ASTM (1990) guideline, wherein eggs could be collected for 6 weeks after 50% of the control hens have laid one egg or until all control pens produced 25 eggs. The latter accounts for the number of eggs a quail or mallard would lay in two clutches in the wild. This collection period corresponds to the optimum period for egg production and data collection, which is the fifth to tenth week after onset of egg-laying (Springer and Collins 1999).

6.2.2 Gamete Viability and Fertilization Rate

Fertility is under direct regulation of the reproductive axis and is measured by the number of eggs set that have viable embryos at first candling. It indicates the impact of xenobiotics on parental reproductive function and is therefore a key endpoint. However, this endpoint provides information only on the integrated effects of both male and female gametic function. To evaluate gender-specific effects on gamete viability when decreased fertility is observed, other methods must be used. For example, the treated birds could be mated with nonexposed birds following the termination of exposure as a fertility trial. This approach is recommended, because the many assays of spermatozoal function, such as sperm concentration and sperm motility, for example, have been poor predictors of fertility in birds (Wishart and Staines 1999; Donoghue 1999).

A limited natural mating fertility trial has been designed for Japanese quail (Reddish et al. 1996). In addition to validating the ability of this modified fertility trial to evaluate individual male reproductive performance, Reddish et al. (1996) also demonstrated the efficacy of iterative least squares analysis proposed by Kirby and Froman (1990, 1991) to examine fertility in naturally mating populations of *Coturnix*. In the limited natural mating fertility trial, the male is mated with an untreated female for 48 h and removed. Eggs produced by the female are then opened each day and examined for fertility. Because sperm in the sperm storage tubules of the female Japanese quail deplete to 50% by the fourth day after copulation and are removed completely by the ninth day, male-specific fertility data can be gathered in a few days (Reddish et al. 1996; Schom and Abbot 1974). In the northern bobwhite, 50% depletion of sperm occurs after 9 days and is complete by 13 days (Schom and Abbot 1974). A similar fertility trial could be conducted for treated females using unexposed males, once the females no longer produce fertile eggs following removal of the original, treated male at 4 to 9 days. Gender-specific effects on sexual behavior, sensitive indicators of endocrine disruption (Section 6.2.3), could also be measured during these fertility trials. When conducting both fertility and behavior trials, it is important to the interpretation of results that birds with reproductive experience be used (Reddish et al. 1996;

Halldin et al. 1999); however, current guidelines require first-year breeders (Section 11.0). Although these trials are relatively short and easily conducted, there is cost added to maintain the untreated birds during a quarantine/acclimation period and the fertility trial, and to maintain the treated birds for up to an additional 18 days. There is additional cost of labor for collecting and inspecting the eggs. Recent development of measures that successfully quantify sperm function in birds may provide a more economical means of determining gender-specific effects of xenobiotics on fertility (Section 6.2.2.1).

Selection of pairs for exposure regimens that start after egg production has begun requires that the pair are producing fertilized eggs. The fertility status of the male is poorly characterized by this method, because with continuous cohabitation and opportunity for multiple copulations, low fertility could be masked. Therefore, allocation of males to the test should be based on tests of gamete viability. Collection of pretreatment data on male fecundity should also be conducted for covariate analysis.

6.2.2.1. Sperm Motility and Morphology, and Fertilization Success. Male fertility has been assessed in avian reproduction toxicity studies by the production of fertilized eggs. Although this endpoint incorporates all the reproductive functions, it is a relatively insensitive endpoint upon which to evaluate the impact of a chemical on the male reproductive system. As discussed above (Section 6.2.2), gender-specific effects cannot be separated in this integrated measure, and therefore, little use of fertility trials, which are considered to be the ultimate test of fertility in poultry (Reddish et al. 1996), has been made in avian toxicity studies. Other traditional measures of semen quality have been less useful in predicting the fertilizing ability of avian spermatozoa, including adenosene triphosphate content of sperm, lipid peroxidation of sperm, sperm morphology, plasma membrane integrity, and cell viability (Reddish et al. 1996; Wishart 1995; Donoghue 1999; Wishart and Staines 1999). Evaluation of sperm quality using measures of sperm number, sperm motility, and sperm morphology to enhance the interpretation of results of avian reproductive toxicity tests and provide information on possible mechanisms of action have been used only sporadically (e.g., Damron and Wilson 1975) and seldom in tests for pesticide registration (Mineau et al. 1994). Common methods for determining sperm concentration (number of sperm per milliliter semen) and sperm volume (sperm concentration times volume of ejaculate) involve counting the spermatozoa in a known amount of semen using a spermatocrit or direct counting hemocytometer, by flow cytometry, or by mounting semen on a hanging drop slide. In addition to ejaculate samples, sperm concentration can be derived from the distal deferent duct at necropsy or from testicular histology. The distal portion of the deferent duct contains 92% of the extragonadal sperm reserve, which is equivalent to the number of sperm produced daily by the testis (Clulow and Jones 1982). Daily sperm production in Japanese quail is about 9.25×10^6 sperm/g testis/day (Lin et al. 1990). Normal sperm concentration is 2.3×10^6/mL in the distal deferent duct and 2.3×10^6/mL in the seminferous tubule of the testis (Kirby et al. 1990). Routine evaluation of avian sperm motility and morphology is performed by either phase contrast or differential interference contrast microscopy (Bakst and Cecil 1991). Scanning and transmission electron microscopy can also be used to examine the ultrastructure of sperm (Thurston and Hesa 1987); however, equipment and supply costs are high for electron microscopy evaluations, and specimen preparation time can extend over several days. Fixation and/or dehydration processes introduce artifacts that also limit the usefulness of electron

microscopy in detecting abnormalities in the ultrastructure of sperm before and after treatments (Bakst 1993). Histological quantification of sperm is discussed further in Section 7.1.2.

Percentage of motile sperm and characteristics of sperm motion have been used by investigators to evaluate potential fertility in poultry (e.g. Wilson et al. 1979; Wishart 1995; Wishart and Palmer 1986; Froman and McLean 1996). Decreased motility of sperm can result form structural abnormalities, loss of mitochondrial function, cytological damage from direct exposure to xenobiotics or hormones during development, or from the effects of abnormal testicular development in response to chemical exposure (Kime et al. 2001). In birds, sperm are immotile prior to ejaculation, limiting collection of sperm for motility samples to those obtained in ejaculated semen (Ashizawa and Sano 1990). Sperm motility, the swirling movement of sperm (swirl method) or the percentage of spermatozoa moving in a forward motion when viewed under high magnification are historically measured by direct count (Wilson et al. 1979). Computer-based measurement of spermatozoa swimming speed as straight-line velocity is a more quantitative and objective, less variable method developed in recent years. Such videographic techniques also provide a permanent record of motility. However, they require substantial technical expertise, and the equipment is expensive. Videographic sperm analysis equipment ranges from about $25,000, without an external negative phase microscope, to $35,000 with a microscope. Basic software for morphology and motility assessments ranges between about $5,000 and $10,000. Other motility measures that are highly correlated with fertility include spectrophotometer techniques based on the rheotactic properties of sperm. For example, in an assay developed by Wishart and Ross (1985), sperm align in parallel under induced flow in a tube, and when the flow is stopped, the light scatters from the sperm changes as the sperm reorient. This change in light scatter is related to the percentage of motile sperm present and the velocity of sperm motion. Another of these methods determines a sperm motility index based on the frequency that sperm within a capillary tube alter a light path in a Sperm Quality Analyzer (Introtech, San Diego, California). These methods are objective, simple, and rapid (McDaniel et al. 1998). However, like most traditional semen assays, they measure one characteristic of sperm (Amann and Hammerstedt 1993) and do not account for the physiological conditions and complexity of fertilization. Motility may not be very predictive of fertility, because oviduct factors also influence sperm movement.

A relatively new approach to analyzing sperm motion simulates a critical step for internal fertilization in the female (Froman and McLean 1996). In contrast to motility measures that determine the percentage of sperm that are moving, this sperm mobility test measures the net movement of a sperm population against resistance, a condition sperm must overcome in the oviduct (Donoghue 1999; Froman et al. 1999). A sperm suspension is placed on top of Accudnez, a dense, nonionic, biologically inert material commonly used in density gradient centrifugation. The absorbance of the Accudenz is recorded after 5 min of incubation at body temperature (41˚C), at which time the absorbance is proportional to the number of sperm that have penetrated the Accudenz layer. The sperm that swim into the Accudenz are highly mobile, directional swimmers. Subfertile males may have a normal semen concentration, but a low number of highly mobile sperm and a high number of immobile sperm, even though they may be technically motile; in motility tests, motile often means “not immotile.” This method has

repeatedly been shown to be a primary determinant of fecundity and highly predictive of fertility and male fitness in poultry (Froman and McLean 1996; Holbserger et al. 1998; King and Donoghue 1998; Rhoads et al. 1998). The method is inexpensive, objective, accurate, and requires little expertise. Interassay CVs for this assay range from 2.6% to 9.2% (Froman and McLean 1996). This procedure has the potential to be a sensitive and economical endpoint in avian reproduction toxicity tests, but needs to be adapted to and validated in quail and under conditions of endocrine and toxin challenge.

The process of fertilization involves not only the transport of sperm, but its storage in the sperm storage tubules of the oviduct, the binding of the sperm to the outer investment, or perivitelline layer, of the ovum at ovulation, and penetration of the ovum (Bakst et al. 1994; Robertson et al. 1998). There have been a number of assays developed and evaluated in recent years to quantify these functions of sperm in an attempt to replace the traditional measures of semen quality which have proven to be poor predictors of male fertility. These new assays quantify the number of sperm that interact with the perivitelline layers of the egg in the infundibulum. Systems measuring the interaction of sperm with the perivitelline layers have proven to be highly predictive of individual fertility in poultry under a variety of environmental stresses. Most of these assays are simple, quick, and noninvasive.

One of the more involved, but automated methods for measuring sperm interaction with the perivitelline layers is an *in vitro* sperm-binding assay (Barbato et al. 1998) that uses microwell plates coated with a solubilized extract of the perivitelline layer from chicken eggs. Sperm suspensions of known concentration are incubated on a microwell plate, the unbound sperm are removed by washing, and the remaining bound sperm are counted. So far ,sperm binding has been shown to be predictive of fertility in several strains of chickens (Barbato et al. 1998; Barbato 1999). A commercial form of the assay (BioPore, Inc., State College, Pennsylvania) is available. The assay requires technical skill and specialized equipment for analysis and ranking of male fertility. Although the method has not been adapted to quail, it has been simplified for use in large-scale poultry flocks (Gill et al. 1998).

The remaining systems that measure the fertilizing capacity of sperm are *in vivo* assays based on 1) the number of sperm that bind to the inner perivitelline layer (IPVL) and undergo an acrosome reaction at fertilization; or 2) the number of sperm that become trapped in the outer perivitelline layer (OPVL) as it is laid down around the IPVL within a few minutes after fertilization. All of the assays involve excising a 1 cm^2 to 2 cm^2 piece of the perivitelline layer from a fresh egg, rinsing the tissue to remove any yolk, and placing the tissue on a slide. To count sperm trapped in the OPVL, indicative of the number of sperm present in the oviduct during fertilization, the tissue layer is stained with DNA-binding fluorochrome and viewed under 400X magnification using a fluorescence microscope. This method requires use of special equipment, and the counting of many fields at high magnification.

For sperm that have bound with the IPVL, released acrosomal enzymes, and hydrolyzed a small hole through which they have passed to reach the oocyte, the tissue is either fixed, dried, and stained with Schiff's reagent (Bramwell et al. 1995; Howarth and Donoghue 1997), or viewed

wet and unstained with darkfield optics (Birkhead et al. 1993). The unstained method involves fewer steps, but must be read within 24 to 48 h. The staining method allows for storage of the tissue and has been shown to result in the identification of more holes (Fairweather 1998). Sperm holes can be counted from any area of the IPVL, though the area directly over the germinal disk has the highest density. Holes over the germinal disk are more easily enumerated and provide data more directly linked to egg fertility (Wishart and Staines 1999). This method takes only a few minutes per slide to prepare and count. It has been evaluated against several sperm quality tests and validated in poultry fertility tests (Robertson et al. 1998; Wishart 1985). The IPVL method also has been successfully used to evaluate the effects of dietary energy on sperm function (Bramwell et al. 1996) and the effects of heat stress on fertility of breeder males (McDaniel et al. 1996). Of the male fertility measures available, the IPVL method is the least invasive, most economical in labor and equipment, requires minimal expertise, provides among the most reliable assessments of fertility, and has been used with success to discriminate between fertility of males subjected to different dietary or environmental conditions. Additional advantages of this assay include 1) the ability to detect differences in fertility between groups of birds before it is detectable in the proportion of fertilized eggs laid (Donoghue et al. 1995); 2) a more accurate measure of fertility status of unincubated eggs than attainable by morphology of the germinal disk (Wishart and Staines 1999); 3) ability to store eggs for several weeks before examination; and 4) ability to substantially shorten standard fertility trials by assessing fertility of the second mating before infertile eggs are laid.

6.2.2.2 Egg Quality. Of the various interrelated functions that contribute to reproductive success in birds, formation of a robust eggshell to protect the developing embryo has been one of the most frequently measured parameters in avian reproductive toxicology. Eggshell quality emerged as a key indicator of reproductive impairment in natural populations of birds from the eggshell thinning phenomenon and associated reproductive failure of several sensitive bird species exposed to OC pesticides in the mid-1900s (Cooke 1973). The mechanisms underlying the daily fabrication of eggshells are not completely understood, but appear to be susceptible to a variety of contemporary environmental chemicals (Haegele and Tucker 1974; Fleming et al. 1983; Ormerod et al. 1988). Since the introduction of the EPA test guideline for avian reproduction studies in 1975, egg quality endpoints, particularly eggshell thickness, have continued to be useful tools in hazard assessment. In a review of 134 avian reproduction studies conducted in support of pesticide registration, eggshell thickness was among those variables shown to be effective in indicating a response to chemical exposure (Mineau et al.1994). Eggshell effects were observed in 17% of the studies.

In addition to providing mechanical protection, a well-formed eggshell prevents water loss, protects against infection, and is a major source of calcium for the developing embryonic skeleton (Lavelin et al. 2000). The functional qualities of the eggshell that provide a protective environment for the developing embryo are its size and shape, thickness, and ultrastructure of mineral and protein. Measures that are used to assess these qualities include shell thickness, breaking-strength, and the number of cracked or soft shells and of eggs without shells. Electron microscopy can be used for detailed examination of the shell. These measures are used to

provide information on the potential for successful development of the embryo and hatch of the chick.

One of the most common assays of eggshell quality, called candling, is the examination of the exterior soundness of the shell. Candling is accomplished by turning the egg, as it is held near a light source, and observing the shell for checks, cracks, and texture (thin spots, ridges). Although it is a simple, inexpensive technique, it is dependent on observation criteria that are undefined in avian reproductive tests. Features such as body checks, which are cracks that occur while the egg is in the uterus or shell gland and that have been repaired with a layer or ridge of deposited calcium, could be scored differently by different laboratories, and by different individuals within laboratories. Depending on the extent of the cracking, checked shells can be weaker than those that are unchecked (Moreng and Avens 1985). Because cracked eggs are removed from the population before incubation, the potential impact of inaccurate or inconsistent scoring on subsequent fitness parameters can be marked. Therefore, a uniform scoring system among laboratories is needed. Other factors, such as handling of the birds, access of the birds to the eggs, housing materials, and slope of the cage floor, can confound the sensitivity of this egg quality endpoint by introducing cracks not caused by treatment.

In an interlaboratory comparison among five separate testing laboratories, quail from the same supplier were used, but the birds were maintained under different husbandry methods. The background proportion of cracked eggs in the controls varied greatly between laboratories, as did the sensitivity of identifying treatment-related effects (Schlatterer et al. 1993). It has also been observed that some quail hens produce clutches with a large number of cracked eggs (Bennett and Ganio 1991). Bennett and Ganio (1991) demonstrated that the distribution of such hens within groups and the rate of cracking per hen could mask chemical-induced effects. Using a typical pattern of cracking in a hypothetical study, in which the same number of pens in each group had the same pen-wide cracking rate, they showed that one-way ANOVA could not detect a true chemical effect in the number of 14-day-old chicks in the low-dose group of their hypothetical study. Because of the potential for masking of chemical-related effects, it is important to the outcome of avian reproduction studies not only to properly identify cracked eggs, but also to evaluate their distribution among hens within groups. Some laboratories use the frequency of cracking as an indirect measure of shell strength (Schlatterer et al. 1993). However, the frequency of cracked eggs is considered to be a poor measure of egg quality and should be used mainly to assure that background cracking rates are within an acceptable range and uniform pattern (Bennett and Ganio 1991).

Shell damage is directly related to shell strength, and shell strength is determined by the organization of the organic matrix, and by the thickness of the shell, measured as calcium carbonate content, particularly on the palisade layer. Thinning of the eggshell can result in egg breakage, egg-eating behavior, or disappearance of eggs from the nest; therefore, measures of eggshell thickness are commonly used to monitor eggshell quality in the wild and in laboratory studies. Cooper (1991) reviewed the relationship of eggshell thinning, eggshell breakage, and reproductive failure in natural populations of birds. He found that eggshell thinning of 10% leads to cracking of the shells and increased embryonic mortality, that extensive egg breakage

occurs when eggshell thinning is greater than 15% to 20%, and that in nature, the latter is associated with population decline (Anderson and Hickey 1972; Lincer 1975; Risebrough et al. 1968; Stoewasand et al. 1971; Spann et al. 1972; Cooper 1991). Cooper (1991) also concluded that thinning of 2% to 5% could cause some loss of eggs, but that it was difficult to discern from background loss levels. There is considerable species variation in the amount of egg thinning observed from similar exposures to chemicals.

Several techniques have been developed to measure eggshell thickness. The three methods in common practice are as follows: 1) determination of the specific gravity of the egg, 2) calculation of a thickness index from the ratio of the weight and surface area of the shell, and 3) a direct measure of the thickness of the shell. All three methods are relatively simple and inexpensive to perform. The specific gravity method is a noninvasive procedure based on determining the egg's density relative to water at the same temperature. Because the specific gravity of a shell is more than twice that of the other parts of the egg (yolk, albumen, and membranes), it has a major influence on the specific gravity of the whole egg. Specific gravity and eggshell thickness are highly positively correlated (Bennett 1992), as are specific gravity and incidence of cracks and breaks. In chickens, hatchability of eggs with a specific gravity of <1.080 was at least 2% less than the hatchability of thicker-shelled eggs. The incidence of embryonic death was also higher in the thin-shelled eggs (Wells 1967). Specific gravity values for *Coturnix* range between about 1.066 and 1.068 (Marks and Britton 1972; Goodman 1965). Similar values are reported for bobwhite eggshells (Mahmound and Coleman 1967).

This method typically involves the preparation of 18 or more salt solutions ranging in specific gravity from 1.030 to 1.090, typically at intervals of 0.0025. The solutions are verified with a hydrometer. Eggs are immersed first in pure water, then into each of the salt solutions, and rinsed in pure water in between test solutions. The specific gravity of the solution in which the egg first floats is recorded. Because this method is accurate only when the egg has small air cells (moisture loss reduces specific gravity measurements), eggs must be measured within 24 h after collection, and consistent timing of the specific gravity determinations are important for reliable results. The specific gravity method has the advantage of not having to sacrifice eggs and thereby reduce the number available for hatching rate measurements. However, the collection time and storage restrictions are inconvenient and result in increased labor, particularly on weekends. Although the specific gravity method could be used in the field (it is used on poultry farms) to sample wild populations noninvasively, it is relatively cumbersome for fieldwork.

Shell thickness indices have been and continue to be used to monitor eggshell quality of a wide variety of wild bird species. Changes in shell thickness of field-collected eggs usually have been detected by one of several indices derived from the weight of the egg and some estimate of its surface area. A small hole is drilled in the egg, the contents are blown out, leaving the membranes intact, and the shell is dried. The eggshell is weighed, and the length and maximum width of the egg are measured. The weight of the shell is then divided by a function of the length and width that is proportional to the surface area of the egg. The oldest index and the one most widely used is the Ratcliffe or Shell Index (Ratcliffe 1967):

$$Wt/LW \qquad (1)$$

where Wt is the weight of the shell, L is the length of the shell, and W is the width of the shell at its widest point. Other indices of thickness include the Nybo-Green index, which is based on early assumptions by Romanoff and Romanoff (1949) and Peakall et al. (1973), and on later empirical data by Pagnaelli et al. (1974) and Hoyt (1979) that the surface area is proportional to L2/3 W4/3 (Nybo et al. 1997; Green 1998), and the Moriarty-Nygard index, which assumes the surface area of the egg is a prolate spheroid with semi-axes L/2 and W/2 (Moriarty et al. 1986; Nygard 1999). Green (2000) compared the three thickness indices using museum collections of eggs for several species of thrushes and found that the difference in coefficient of variation among the indices was very small (e.g., 7.393-7.597 for the European blackbird, 5.872-5.909 for the Mistle thrush), indicating that the ability of these indices to detect a decrease in shell thickness was about equivalent, at least for the species measured. The preparation time for this egg quality assay is about equivalent to that of the direct method discussed below, but the actual measurement time is much less labor-intensive and not as subject to fatigue effects and individual variation in sample point selection and measurements.

Current guidelines for avian reproduction tests (EPA 540/9-82-024; OECD 206) support the use of direct eggshell thickness measurements to evaluate the effect of a chemical on the hormone-mediated process of shell mineralization. This method consists of cutting an egg open around its equator, emptying the contents, and drying the eggshell with its membranes, after which the shell thickness is measured with a micrometer. It is important to consistently obtain thickness measurements from one specified region of an egg, because shell and membrane thickness can vary significantly along the egg (Dirksen et al. 1991). The equator of the egg is selected as the area from which the measurements are taken, because it represents a wide band of uniform thickness. Also this area is the traditional measurement site used by early investigators of egg quality effects of dichlorodiphenylethylene (DDE) and other environmental contaminants (e.g., Koeman et al. 1972; Newma 1979). Drying times vary from 1 day to 1 week under ambient conditions, although 48 h of drying appears to be used most often. A number of measurements between three and nine should be selected as standard per shell for each test. Although no formal study has been conducted, several researchers believe that there can be substantial differences in values obtained by different staff for the same shell, and that less variable data are obtained when a single staff member conducts the measurements throughout the course of the study. Variation is thought to arise from the way in which a micrometer is held or sample areas on the shell are selected. For example, it has been noted that areas where the underlying membranes have been disrupted during preparation of the egg differ greatly in thickness from surrounding areas with intact membranes. Because fatigue of the staff can affect the quality of data during a long session of measuring shell thickness, some laboratories begin each test lot with control eggs. Even small improvements in data quality are important in light of the relatively few eggs that are tested for eggshell thickness during reproductive toxicity tests.

Although thickness contributes to the overall strength of the shell, it accounts for only a small fraction of the shell resistance to mechanical stress (Lavelin et al. 2000). Researchers have used

two assays to measure the resistance of eggs to mechanical stress. One technique measures the shear fracture force required to puncture the shell (Stevenson et al. 1981). This puncture can be repeated several times on each egg to obtain an average puncture force value. However, it has the drawback that it does not represent the resistance to forces encountered in nests in the field and is therefore seldom used in avian reproduction studies to evaluate shell strength. The second egg strength assay measures the compression strength of the shell that more directly represents the mechanical stress encountered during natural incubation. In this method, the tensile fracture force is measured with a universal testing instrument, wherein an egg is compressed at the equator between two stainless steel surfaces. The compression on the egg and the load at which the egg failed are recorded. Studies comparing the eggshell thickness and compression strength methods with both laboratory-treated and field-collected eggs indicate that the compression-breaking-strength method can detect shell damage at lower exposure concentrations than the shell thickness assay (Cooke 1979; Snyder et al. 1973; Carlisle et al. 1986; Bennett et al. 1988; Henny and Bennett 1990). Scanning electron microscopy has been employed to examine the integrity of shell ultrastructure in eggshells that were evaluated by both shell-thickness and breaking-strength assays. Ultrastructure defects were observed in shells that were of normal thickness but were identified as weak by the compression-strength assay (Bennett et al. 1988, Henny and Bennett 1990). In these eggshells, the mammallae, an array of rounded cones that forms the foundation layer of the shell on which the remaining crystal growth occurs, were poorly formed and irregular, indicating that resistance to breakage is a direct function of eggshell integrity rather than of than thickness, and that compression-breaking strength can provide an indirect measure of ultrastructure integrity.

Although more sensitive than the thickness assay in identifying treatment effects, the breaking-strength test appears more variable than shell thickness. The coefficient of variation for the compression test data in the Bennett et al. (1988) study was 12% to 16% for pretreatment eggs compared with 9% for thickness measurements on the same shells. Another drawback of the compression test is that it cannot be performed on cracked or soft-shelled eggs, whereas the thickness assay can be used on eggs in those conditions. The use of both methods has been encouraged by a number of investigators to better assess the potential damage to eggshell quality from dietary exposure to xenobiotic chemicals, in general (Carlisle et al. 1986; Bennett et al. 1988; Henny and Bennett 1990). It is particularly important in the context of an avian reproduction toxicity test to maximize the shell quality information obtained from the relatively few eggs tested relative to the number produced.

Although the compression-breaking-strength method is more sensitive than the thickness assay, the high up-front cost of the equipment to perform this test has limited its use. However, once equipment is established, there are labor savings in using the assay. Cost of universal materials-testing instruments ranges from $10,000 to $30,000, depending on manufacturer, computer and software options, and accessories. Labor hour estimates from two commercial testing laboratories that conduct both eggshell thickness and shell strength are about 8 h per 200 eggs plus an additional 4 h of preparation time per week for shell thickness measurements. In contrast, about 4 h of labor are required to measure the breaking-strength of 200 eggs, with little to no preparation time.

Recent studies indicate that shell strength is positively correlated with the matrix proteins that pervade the mineral phase of the avian eggshell and influence mineralization. Several eggshell matrix proteins that have the potential to be biomarkers of shell quality have been identified (Hike et al. 2000). These researchers have clearly identified a high correlation between expression of one of the proteins in the mammillae, and shell strength and ultrastructure integrity. It is likely that immunochemical analysis of the eggshell matrix will ultimately provide a rapid, simple, and highly sensitive measure of eggshell quality in the future.

6.2.2.3 Hatching Success. Hatching is a critical developmental stage that is vulnerable to disruption. It has been shown to be sensitive to a wide array of chemicals (Hoffman and Heinz 1988; Heinz et al. 1989; Rice and O'Keefe 1995; Hoffman 1978; Coon et al. 1979; Hoffman 1990) with consequent increases in mortality. Therefore, hatching success is a key endpoint in avian reproduction toxicity tests. There are a number of factors that reduce hatchability. In addition to handling trauma, incubation conditions, and other husbandry issues, hatchability is affected by poor eggshell quality, nutrition of the hen, which corresponds to nutrient exhaustion of the embryo, and teratogenic factors. Embryo viability and subsequent hatching success also provide information on the effects of *in ovo* deposition of chemicals on embryo development. These measures are highly correlated in reproductive toxicity tests with chick survival to Day 14 (Mineau et al. 1994). Typically, embryo viability is evaluated at both early and late periods of development, providing information on fertility and embryo toxicity, respectively. Viability at these two time periods is generally reported separately; however, a combination of the early and late embryo viability variables is a more effective indicator of effect (Mineau et al. 1994).

Embryo viability and the corresponding hatching success are also related to eggshell porosity, which affects gas exchange and rate of water-loss in the egg. Peebles and Marks (1991) demonstrated that increased eggshell permeability was associated with embryonic death and decreased hatchability in Japanese quail selected for meat-production growth. Altered eggshell quality could be one of the factors contributing to differences in reproductive parameters of different strains of Japanese quail, and underscores the need to standardize strain selection.

6.2.2.4 F1 and F2 Post-Hatch Survivorship. Post-hatch survivorship is a primary production endpoint that integrates the following:

- *fecundity* number of eggs laid
- *fertility* proportion of eggs laid or set that are fertile
- *embryogenesis* proportion of live embryos of those fertile
- *hatchability* proportion of eggs with embryos that hatch
- *chick viability* proportion of hatchlings that survive to 14 days old.

Although this endpoint provides an overall measure of reproductive capacity/success per pair or per pen, CVs for this parameter are high, and the power of tests to detect 20% reduction in the number of 14-day survivors of quail is lower than that for the other reproduction endpoints (Springer and Collins 1999). To attain adequate power to detect effects on this endpoint, a significant increase in the number of replicates would have to be incorporated into the test design, if ANOVA methods were used.

6.2.3 Changes in Breeding Behavior

Appropriate, timely breeding behavior is an important component of reproductive success in birds. Evidence of altered breeding behavior in wild birds exposed to environmental pollutants has been reported (Peakall and Fox 1987; Fox 1993; Thaxton and Parkhurst 1973). Recent studies with endogenous and exogenous steroids indicated that exposure to ecosteroids at critical periods of a bird's life can cause profound, irreversible changes in these critical behaviors (Berg et al. 1999; Halldin et al. 1999; Eroschenko et al. 2002 ; Balthazart and Surelemont 1990a; Panzica et al. 1996; Panzica et al. 1999).
Sexual behavior in the Japanese quail has been well described (Schein and Carter 1972; Sefton and Siegel 1973), and it includes courtship behavior, such as crowing and strutting, and mating behaviors, such as copulation. In male birds, sexual behaviors are androgen-dependent (Adkins and Adler 1972; Adkins 1977), and there appear to be varying thresholds of hormonal stimulation (Ottinger and Brinkley 1978). For example, onset of crowing occurred at serum testosterone levels of 3.3 ng/mL, whereas 4.7 ng/mL was required for the initiation of mating behavior.

Testosterone has been shown to affect behavior by its action on specific regions of the brain. The lateral septum, medial preoptic nucleus (POM), and bed nucleus of the stria terminalis (BST) are sexually dimorphic structures in the quail brain that are involved in the activation of breeding behaviors (Halldin et al. 1999; Balthazart and Surelemont 1990a, 1990b; Panzica et al. 1996; Panzica et al. 1999). Hormonal manipulation of juveniles and adults results in profound morphological changes in the POM (Panzica et al. 1994) and subsequent changes in copulatory behavior in the Japanese quail (Halldin et al. 1999). During embryogenesis, exposure to estrogens or estrogen agonists organizes areas of the POM in male birds in a nonmasculine manner, resulting in irreversible depression of copulatory behavior in the adult. Such *in ovo* exposure has been shown to cause significant depression of male sexual activity at concentrations well below those that affect plasma testosterone levels, body weight, or the gonadosomatic index (Berg et al. 1999). Eroschenko et al. (2002) demonstrated that at maturity, male Japanese quail that had been exposed to methoxychlor, a metabolite of methoxychlor, or to 17 β-estradiol *in ovo*, displayed significantly altered sexual behavior, such as decreased copulation attempts and lengthened mounting latency, although cloacal gland size, and the weight and histological morphology of their testes were normal. These data indicated that prenatal exposure to even weakly estrogenic compounds could result in a permanent feminization of the brain of male Japanese quail.

Measurement of copulatory behavior is well studied in Japanese quail, because this species has been used for many years as a model for studies of testosterone control of breeding behavior (Mill et al. 1997). Copulatory behaviors are governed by the aromatization of testosterone to estrogen (Balthazart et al. 1995). The behavior is well defined and follows a particular sequence of neckgrab, mount attempt, mount, and cloacal contact movement (Halldin et al. 1999). It is easily measured in the laboratory by housing male birds singly and then recording specific responses following introduction of a receptive female into the cage. These measured responses are indicative of sexual motivation and the ability to exhibit reproductive behavior. They include

latency to mount, mating attempts, and completed matings. Tests that can be completed in 2 to 5 min for each bird on as few as 2 to 5 consecutive days have been shown to accurately reflect male reproductive behavior (Halldin et al. 1999). However, size of the test arena can affect the results: small arena size (50 cm x 60 cm) greatly enhances response (Riters et al. 1998), probably as a result of the proximity of the female to the male bird. Care must be taken to reduce observer bias by conducting the tests such that the observer is unaware of the treatment associated with the test subjects. Further, it could be important to select behaviors for measurement that are less dependent on the receptivity of the female, such as mount attempts and cloacal contact movement; they are better measures of male consummatory sexual behavior (Halldin et al. 1999).

Courtship has also been studied (Ottinger et al. 1982). Like other sexual behaviors, it appears to follow a cyclic pattern (Wada 1982; Ottinger et al. 1982). When behavioral tests are used as endpoints for reproduction toxicity studies, the cyclicity of the behavior must be taken into account so that behaviors are measured within the appropriate and same activity period during the day (Halldin et al. 1999).

Appetitive sexual behavior and copulation behavior of the male quail appear to be under similar endocrine control: aromatization of testosterone in the brain (Balthazart et al. 1997, 1995). These behaviors indicate male interest in the female, and they include precopulatory responses, such as approaching and remaining near a female. Two methods have been used by a number of researchers to measure appetitive sexual behavior in Japanese quail treated with steroids. One index of response measures the duration and number of times a male quail stands in front of a narrow window that allows him to see a female in an adjoining cage. This proximity response is learned behavior and is only displayed in males that have copulated with a female (Domjan and Hall 1986; Riters et al. 1998). The second method is independent of sexual learning, although Riters et al. (1998) showed a stronger response in experienced males. It measures the rhythmic contraction of the cloacal sphincter of a male provided visual access to a female. Cloacal sphincter contractions occur during the production of foam that is transferred to the female cloaca during copulation (Cheng et al. 1989a, 1998b). Simple visual counts of contractions are made by an observer who views the bird through a mirror positioned below a glass floor, such as that of an aquarium as test chamber.

To date, breeding behavior appears to be among the most sensitive endpoints measured for birds exposed to estrogen or estrogen-agonists (Halldin et al. 1999). Male copulatory behavior has been shown to be a highly sensitive indicator of *in ovo* endocrine disruption and is the most commonly tested behavioral endpoint in endocrine effects tests. Currently, the endpoint of copulatory behavior is being compared in both bobwhite and quail at the University of Maryland (M. A. Ottinger, personal communication, 2002). Given the sensitivity of this endpoint and its

ecological relevance, male copulatory behavior should be incorporated into avian reproductive toxicity tests to evaluate EDCs in F1 males.

6.3 Neurological/Central Nervous System Impairment Tests

Endpoints that are indicative of central nervous system (CNS) effects and/or hormonally-mediated toxic responses measure behaviors, such as open-field motor ability, panic and separation responses, and hearing and location skills. These behaviors reflect integrated response to toxic or hormone insult. Open-field tests provide indication of motor problems associated with neural or CNS impairment and can be conducted at any stage of maturation, from juvenile to adult. Runway tests are often conducted with young chicks to take advantage of the chicks' need to group with conspecifics. An individual chick is placed on one end of a runway opposite an open cage containing conspecifics. The course, the time to rejoin conspecifics, and behaviors such as defecation, inquisitiveness, and tonic immobility, are recorded. These tests are repeated as the chicks age to track performance improvement and maturity, with less need to seek conspecifics. In a recent test using Japanese quail, chicks hatched from parents fed an endocrine disrupting chemical, methoxychlor, displayed immature behaviors longer than did controls, suggesting a slower maturation in response to parental exposure (M.A. Ottinger, personal communication, 2002). The test was ineffective in measuring the response of northern bobwhite chicks, because they did not adapt well to the test paradigm. In runway tests using maternal calls as the approach incentive, ducklings treated with DDE were more responsive than control ducklings to the calls (Heinz 1976), whereas treatment with methylmercury resulted in hyporesponsive ducklings (Heinz 1979). The opposite was found in runway avoidance tests with a fright stimulus. In the avoidance tests, methylmercury treatment resulted in hyper-responsive ducklings, whereas ducklings treated with DDE were hyporesponsive to the stimulus (Heinz 1976, 1979). In contrast, activity level and exploratory behavior in an open-field test were unaffected by DDE exposure (Heinz 1979). Runway tests appear to be sensitive in some species and are relatively simple to perform, but require expertise in interpreting results. Additional work is required to verify which behavior, or set of behaviors or stimuli, are most effective and constitute an adequate endpoint for a wide variety of chemicals. The most convenient age to test the chicks is14 days, when the F1 and F2 survivors are terminated. The determination of the sensitivity of the behavioral assays at this age should be emphasized.

7.0 ASSAY ENDPOINTS: PHYSIOLOGICAL

This section describes the physiological endpoints that can serve as direct measurements of endocrine activity in an avian two-generation reproduction toxicity test. Emphasis is given to endpoints recommended by EDSTAC (EPA 1998) and those compiled by OECD. Additional endpoints were added from the literature review. The physiological endpoints include morphological and histological evaluations, brain chemistry, and plasma and fecal steroid analysis. These endpoints are listed in Table 7-1 in relation to the life stage at which the endpoint can be measured. The potential underlying endocrine mechanisms (estrogenic, androgenic, and thyroidogenic) are also provided.

Table 7-1. Physiological Endpoints Specific to Endocrine Active Substances[a]

Physiological Endpoints	Critical Life Stages for Evaluation of Impact				Type of Endocrine Activity		
	Embryo genesis	Early develop-ment	Sexual maturation	Egg-laying	Estrogenic	Androgenic	Thyroido-genic
For Parents and Adult F1							
GROSS MORPHOLOGY AND HISTOLOGY							
size and weight of testes, ovaries, thyroid, adrenals, brain, oviduct		yes	yes	yes	+/–[b,c]	+/--	+/--
histology of thyroid, adrenals, gonads, and brain		yes	yes	yes	+/--	+/--	+/--
testicular spermatid counts and morphology, sperm mobility test, perivitelline layer sperm				yes		+/--	
gross anomalies of the genital tract		yes	yes	yes	+/--	+/--	
cloacal gland area		yes	yes			+/--	
PLASMA AND FECAL/URATE HORMONES							
steroid hormones (estradiol, testosterone, corticosterone)		yes	yes	yes	+/--	+/--	
vitellogenin (males only)		yes	yes	yes		+/--	
thyroid hormones and TSH		yes	yes	yes			+/--
BRAIN CHEMISTRY							
GnRH		yes	yes	yes	+/--	+/--	
catecholamine		yes	yes	yes	+/--	+/--	
aromatase		yes	yes	yes		+/--	
foam gland test			yes			+/--	
presence of medullar bone			yes	yes	+/--		
For F1 and F2 Chicks							
gonad weights	yes	yes			+/--	+/--	
oviduct weights and differentiation		yes			+/--		
wing and bone length		yes				+/--	+/--
skeletal X-ray		yes				+/--	+/--
thyroid weight and histology		yes					+/--
plasma sex steroids		yes			+/--	+/--	+/--

a) Table from Bennett et al. (2001).
b) + indicates positive activity (e.g., estrogenic, androgenic, thyroidogenic).
c) – indicates negative activity (e.g., antiestrogenic, antiandrogenic, antithyroidogenic).

7.1 Organ Growth and Morphological Changes

Measures of growth and morphological changes of reproductive organs and their accessory structures are primary endpoints of xenobiotic impact on the development and integrity of reproductive tract. The gross measures of organ weight and gross morphology are among the most economical of the endocrine-sensitive endpoints. Microscopic determination of gonadal abnormalities, gamete production, and steroidogenic capability are among the most sensitive measures that can give insight into the mechanism of action or target structures of chemical-induced injury or endocrine imbalance.

7.1.1 Development of Gonadal and Accessory Structures

Gonad weight is a rapid quantitative index that reflects the effect of a test substance on gonadal development and reproductive condition. For example, weights of testes and epididymes are often used as indicators of possible alteration in androgen status and a correlation between testis weight and the number of germ cells in the testis has been demonstrated (Zenic and Clegg 1989). Yet, testicular weights are not as sensitive as sperm counts in assessing reproductive capacity and are compromised by edema (Thomas and Thomas 2001). Ovary weight in growing and mature birds is a good measure of endocrine and reproductive condition. A companion measure of reproductive status in female quail, considered by some authors to be a better predictor of female maturation than ovary weight, is oocyte diameter. One version of this endpoint is the measurement of the diameter of the largest ovarian follicle prior to first egg laid (Phillips et al. 1997); another is the mean number of oocytes per female that show initiation of rapid growth–that is, oocytes greater than 4 mm in diameter and yellow in color (Thanton and Parkhurst 1973). A pattern of normal ovarian development in Japanese quail has been described from hatch to adulthood by several investigators (e.g., Pageaux et al. 1984; Lucy and Harshan 1999); however, the reported timing of growth events varies significantly among them.

For example, Lucy and Harshan (1999) reported that rapid growth of the oviduct of Japanese quail begins between 30 and 40 days of age and attains adult weight and functional maturity between 50 and 60 days of age. By comparison, Pageaux et al. (1984) reported that rapid growth of the oviduct of Japanese quail began between 21 and 28 days of age, reaching adult weight by 45 days. Such differences underscore the variation management practices between laboratories and/or in sexual maturation rates found among the various breeding stock of Japanese quail. The characteristic response of the oviduct to estrogen is an increase in wet weight, water content, and glycogen (Bitman et al. 1968). Differentiation of oviduct segments (infundibulum, magnum, isthmus, shell gland, and vagina) can be used as a measure of the maturation (Lucy and Harshan 1999) and can be used as another easily obtained biomarker for delayed or accelerated maturation at necropsy. Incidence rates of right oviducts in F1 and F2 chicks indicate abnormal endocrine signal, and in adult birds, is important to measure, because it is a condition that is associated with reduced fertility in Japanese quail (Rissman et al. 1984).

A characteristic feature of sexual maturation in birds is the asymmetry of their gonads. In most species, the right ovary does not develop, and the right testis is often smaller than the left (Perrin

et al. 1995). A ratio of testis weights, left divided by right, is often used to characterize the effect of xenoestrogens in reducing the size of the right testis (Rissman et al 1984).

Organ weights in toxicity studies are typically recorded in one of three formats, as the absolute or wet tissue weights, or as somatic or brain indices. An organosomatic index is the absolute weight of the organ normalized to the weight of the animal. It provides a general measure of maturity and reproductive condition. However, this approach assumes that the ratio of organ weights to body weight describes the entire relationship between the two variables and does not account for positive or negative growth of an organ with respect to the overall body weight (Salsburg 1986). Therefore, normalizing organ mass to body weight if changes in body weight occur, for example, could mask the effect of a toxicant on the size of the gonads (Ballantyne 1999). If the strain of quail experiences photoperiod drift and if some individuals within groups therefore cannot be synchronized, then a large variability will be manifested in this endpoint (Grossmen et al. 1982). Statistically, a better measure of organ growth is multivariate analysis of covariance of the gonad and body weights (Salsburg 1986; Weatherly 1990). Regression analysis is performed on the body weight and gonad weight data of each group. A weighted average is calculated from the quotient of the gonad weight and the slopes for each treatment group. Gonad weight is then adjusted by the average body weight.

Because of the potential problems with normalizing organ weight by body weight of the animal, some investigators report gonadal weights as a ratio of the organ weight to the brain weight. Brain weight is used in the index, because this measure is usually very stable, and chemicals that cause a change in body weight typically do not affect the brain weight of the animal (Ballantyne 1999). Reporting gonad or other organ weights relative to brain weight requires that the brain be removed with precision. In particular, the point at which the brain is severed from the brain stem must be clearly established in the necropsy protocol and then strictly followed.

Developmental landmarks in F1 and F2 chicks may be used to assess physiological age of a growing animal. Morphological features can be evaluated during early weeks of growth, and the developmental landmarks must be clearly identifiable and directly related to normal ages or stages of maturation. Potential avian developmental landmarks include hatch date, age of female when first egg is laid, age of male when foam gland function begins and gland size increases, and age at which sexually dimorphic plumage develops. A summary of developmental landmarks and approximate ages is provided in Table 7-2. Histological examination of major tissues can also provide information on toxic effects of test substances on maturing organs and tissues (Section 7.1.4). Embryonic histology relative to gonadal development is discussed in this section, because it is well described for the perihatch period, which is usually 2 days before hatch, it has application to field exposure assessment, and it decreases with age posthatch.

Table 7-2. Gross Landmarks of Sexual Differentiation in Japanese Quail and Age of Appearance[a]

Parameter	Mean Day of Onset	Maximum (day)
testes weight (mg)	22	49-57
sexually dimorphic plumage[b]	21-25	–
crowing (per 30 min)	33	50
age of male when foam gland matures	32	50 (maximum area)
age of onset of egg production	42	77[c]
mating attempts (per 5 min)	34	52
cloacal contacts (per 5 min)	37	81

a) Data from Ottinger and Brinkley (1979b).
b) Estrogen-dependent.
c) About 3-5 weeks after first egg laid.

7.1.2 Histopathology in Juveniles

Light and electron microscopy of immature gonadal tissue can be used to detect changes in gonadal morphology and ultrastructural sterodiogenic capability (Abdelnabi et al. 2000).

Because the avian female is heterogametic (Z/W), estrogen is important for differentiation to a phenotypic female (Andrews et al. 1997), and inhibition of estrogen synthesis will result in genetic females with male phenotype (Elbrecht and Smith 1992). Exposure *in ovo* to estrogenic compounds gives rise to males with feminized sex organs, such as the presence of Müllerian ducts and ovary-like tissue in the left testes, and females with Müllerian duct malformations, such as the persistence of a right Müllerian duct or hypertrophied ducts. The most sensitive of these endpoint malformations is the transformation of the left male gonad into an ovotestis. Significantly increased incidence of male embryos exhibiting an ovotestis was observed at doses as low as 0.7 ng/g egg for endogenous or exogenous estrogens (Berg et al. 1999). The ovotestis results from the juxtaposition of the male medulla and an ovary-like cortex, each characterized by its own histological features. The scoring criterion for an ovotestis is the appearance of oocyte-like cells in meiotic prophase in the male testicular cords (Berg et al. 1999). The degree of feminization of the left testis has been quantified by comparing the abnormal area with the total area using image analysis. The right testis in treated male embryos is markedly reduced (Perrin et al. 1995). Genetic females treated *in ovo* with estrogens have enhanced cortical proliferation of the ovary and a severe reduction of the rudimentary right gonad. Gonadal dissymmetry is not conserved after hatching, but decreases with age, disappearing prior to sexual maturation. After hatch the germ cells of the ovotestes enlarge and are enclosed in follicles, but ultimately are resorbed between 3 and 5 weeks of age (Scheib and Reyss-Brios 1979). Boss and Witschi (1947) reported that *in ovo* exposure of herring gulls (*Laras argentatus*) to stilbesterol resulted *in ovo* testes that persisted for 4 years, but the gulls were injected with stilbesterol

weekly after hatch. Halldin et al. (1999) also found that embryonic exposure to diethylstilbestrol or ethinyletradiol did not result in observable histological changes in the testes of 8-week-old male quail, but pointed out that persistence of ovotestes into adulthood could be dose-dependent.

It should be noted that transformation of the left testis into an ovotestis has been observed in untreated control embryos. In a study conducted by Berg et al. (1999), about 18% of the controls had ovary-like tissue in the left testis. In female quail, Müllerian duct malformations from *in ovo* exposure to estrogenic compounds appear to be conserved into adulthood (Rissman et al. 1984; Adkins-Regan et al. 1995).

Because ovotestes and Müllerian duct malformations are sensitive measures of endocrine disruption from *in ovo* exposure and are likely to persist in 14-day-old chick, these tissues should be collected from at least one of the weekly batches of the F1 and F2 surviving chicks. Weights of the gonadal tissue and its appearance at necropsy should be recorded. The tissue should be preserved for histological examination and the gonadal tissues from control chicks and chicks of the high dosed parents evaluated histologically. Additional dose groups can be examined if warranted by the presence of abnormal gonadal tissue in the high-dose chicks.

Having information on the dose and embryonic/chick histological response to the chemical would provide exposure information and comparison for field studies or cases in which egg samples of wild birds can be collected.

7.1.3 Histopathology in Sexually Mature Individuals

Histological methods are sensitive indicators of gonadal function and pathological processes in animals and can provide information on the site of action of a xenobiotic. However, usefulness of these observer-based methods to quantify effects in avian reproduction toxicity tests is limited by lack of a uniform classification system and codification of evaluation criteria. Standardization of structural measurements, such as tubule lumens, randomization of selected structures, such as tubules, for measurement, and scoring criteria would provide repeatable interpretation of tissue changes induced by xenobiotics and endogenous hormones in birds that can be verified by different investigators. For example, routine histological evaluations detect testicular damage only when there is severe depletion of the seminiferous epithelium, obvious cellular degeneration, or sloughed cells in the seminiferous tubule lumen. Less prominent, though reproductively important lesions could be identified by morphometric measurements of the testicular structures. Of particular importance in the evaluation of testicular injury is the use of seminiferous tubule stages (Creasy 1997; Lin and Jones 1993). Quantification of cell staging involves examining cross-sections of seminiferous tubules to determine the frequency distribution of distinct, sequential associations of developing germ cells, which could be identified as stages of proliferation and differentiation. In the Japanese quail, these stages have been well characterized and are arranged in a helix that extends along the length of the seminiferous tubule (Lin and Jones 1990). The duration of the stages is a time-specific process that ranges from 2.5 h to 15.5 h in Japanese quail (Lin et al. 1990). Therefore, the abundance of any given stage is directly proportional to the duration of the stage. Quantitative and semiquantitative methods devised for the assessment of the effects of xenobiotics on testicular

germ cells involve measurement of the cross-sectional area of semiferous tubules and the ratio of the germ cell to the Sertoli cell, and counting the number of spermatids per seminferous tubule (Creasy 1997).

In addition to the codification of lesions or structural measurements, components of histological examinations that need to be standardized to avoid artifacts that can interfere with the evaluations are 1) handling of unfixed reproductive tissues; 2) the tissue embedding and sectioning processes; 3) selection of the chemical fixative protocol and stains; 4) sectioning procedures; and 5) the codification of lesions or structural measurements. Handling-artifacts in testes are common and result from dropping testes on weighing scales or from excessive manipulation of the tissue with forceps (Foley 2001). The common fixatives used in histology of reproductive tissues are 10% neutral buffered formalin and Bouin's solution. Tissue shrinkage and staining properties are different for these two fixatives. Formalin fixation, though routinely used, results in artifacts in the germinal epithelium of the testis when used prior to embedding in paraffin. Bouin's fixative provides superior preservation of cellular detail in tissues such as the testis (Foley 2001). There are a number of disadvantages of using Bouin's fixative: critical timing of tissue trimming and processing, and storage of fixed tissue, for example. Therefore, protocols using a combination of initial fixation of the testes in formalin followed by immersion of trimmed sections in Bouin's solution have been developed to minimize the use of Bouin's fixative and retain the ease of collecting tissues in formalin–a single, multipurpose fixative (Foley 2001). For the most precise staging of the seminferous epithelium in the testis, the germ cell acrosome should be stained using periodic-acid Schiff's method (Clermont 1972). Formalin fixation can be used to examine the seminferous epithelium if it is then embedded in plastic and stained with touluidine blue (Russel and Frank 1978; Ulvick et al. 1982). Hematoxylin and eosin or touluidine blue are common stains applied to gonadal tissue. Each provides a different view of tissue structure. Use of plastic embedding provides greater support of the tissue and thinner sections. Samples for histopathological examinations should be collected from the same area of each organ and serially sectioned along the long axis of the gonad. Samples should be examined "blind," without knowledge of the treatment.

Because of their large volume of yolk, mature oocytes and ova have been difficult to preserve for microscopic examination. Immersion fixation of follicular oocytes in paraformaldehyde and glutaraldehyde fixative followed by a second immersion in fresh fixative to assure penetration and adequate preservation provides the best fixation of the ovarian follicles for light microscopy. For electron microscopy, tissues are trimmed further and placed in osmium tetroxide before sectioning (Bakst 1993).

Preparation of specimens from the oviduct or excurrent duct for light microscopy are routine. However, the secretory function of the oviduct appears to develop sooner than the organs gross differentiation (Reddy et al. 1992); therefore, use of histochemical or immunocytochemical techniques to detect oviductal secretion of fibrous albumen proteins could be used to measure the maturation of the organ. These oviductal secretions can be problematic when one is examining the tissue with scanning electron microscopy, because they can obstruct the luminal surface. Similarly, a clot of sperm can obscure the luminal surface in the excurrent ducts of the testis.

Procedures are available that reduce the incidence of these interferences (Bakst and Howarth 1975; Kirby et al. 1990).

Lectin probes have been used to distinguish the stages of avian spermatogenesis, by binding to a terminal saccharide of a glycoconjugate expressed on either a spermatogonia, spermtocyte, spermatid, or Serotoli cell (Bakst and Cecil 1988). However, fixatives can mask this binding. Unfortunately, the fixative did not mask lectin binding, but provided adequate tissue preservation contains merucuric chloride (sublimate formalin). Substitution of less hazardous zinc chloride could be possible, but has not been evaluated.

There are fewer professional pathologists with experience in evaluating avian tissues than of those with experience in mammalian histology. The level of quanitification and codified description of reproductive organs for GLP evaluations that has developed over the years for mammalian toxicity testing is lacking in avian applications. In particular, familiarity with the stages and cycle of the seminiferous epithelium of the testis that is essential to injury assessment has not been applied to avian toxicity studies. However, the stages of the germ cells in the Japanese quail and poultry are well described and used to evaluate fertility and the impact of endocrine-moderating chemicals on spermatogenesis. Histological examination by professional pathologist in the USA costs from $8 to $16 per tissue, when conducted under GLP criteria for mammalian studies.

7.1.4 Histopathology and Organ Weights of Nonreproductive Tissues

The thyroid, adrenal gland, and brain are potentially affected by a number of EDCs. They can be analyzed by classical histological techniques for evidence of abnormal structure that generally results in dysfunction. However, histopathological examination of tissues from birds exposed to potential reproductive toxins in avian reproduction toxicity tests has been limited (Mineau et al. 1994). New immunological methods can provide more objective and precise information of changes in morphology and function in tissues. A review of mammalian reproduction studies indicates that the adrenal gland is the most susceptible of the endocrine glands to chemical-induced lesions (Ribelin 1984). About 40% of the studies reporting histological effects in endocrine tissue were compounds that damaged adrenal glands. The thyroid was affected by15% of the compounds. As noted above, methods of tissue handling, fixation, staining and lesion scoring should be clearly established to help standardize evaluations.

7.1.4.1 Brain. In recent years, several research groups have been investigating the effect of steroids on the innervation of the septopreoptic region of the hypothalmus of birds. The medial preoptic nucleus and lateral septum are sexually dimorphic and highly involved in the activation of adult sexual behavior of Japanese quail (Halldin et al. 1999; Balthazart and Surelemont 1990 a, 1990 b; Panzica et al. 1996). Hormonal manipulation of juveniles and adults results in profound morphological changes in these areas of the brain, including changes in the density of vasotocin fibers and the number and size of aromatase-containing neurons (Panzica et al. 1994). These changes occur in response to photostimulation and aging, but also as a result of exogenous steroid exposure (Panzica et al. 1996). Although examination of the septopreoptic tissue can provide information on the underlying mechanisms of reproductive depression, measures of the

behaviors activated by this area are highly sensitive to changes in steroid concentration and more economical to perform. Fixation of the tissue and the immunochemistry on serial sections of the septopreoptic region of the hypothalamus are relatively lengthy processes. The specific antibody for the immune reaction is not commercially available, and quantitative determination of the density of the immunoreactive structures requires computerized image analysis.

7.1.4.2 Thyroid. The close link between secretory function of endocrine tissue, such as the thyroid, and proliferative activity agrees with the frequent observation in toxicity studies that a common response to excessive stimulation is endocrine hyperplasia neoplasia (Pawlikowski 1982). Generally, therefore, organ weights should be obtained prior to histological examination of these organs. However, excising and trimming firmly bound organs such as the thyroid gland for organ weight determination is not advisable because of the risk of disruption of histological sections. The thyroid is a small, firmly attached organ deeply embedded in tissue and is difficult to excise and trim. Not only does this introduce variation in organ weight measurements, but the degree of handling required can so strongly affect the quality and orientation of sections as to offset the benefit of organ weight data. Therefore, thyroid tissue should not be excised for organ weight, but removed with minimal handling for histological preparation (Greaves 1999). A means of obtaining thyroid weights is to excise the organ within the surrounding tissues and fix it in preservative prior to trimming. Subsequently, trimming and weighing the preserved gland will minimize damage to the gland and allow for more consistent weights. Thyroid hypertrophy can then be quantified histologically. Enlarged thyroids may reflect either a hyper- or hypofunctioning gland (Wentworth and Ringer 1986). Initially, examination of the thyroid should be completed on the controls and high-dosed groups. Histopathology of the thyroid can be conducted on other groups if warranted by the detection of lesions in the thyroid tissue of the high dosed birds. Immunocytochemical techniques have superseded trandioal tinctorial stanis for the demonstration of poylpetide hormones (Greaves 1999).

7.1.4.3 Adrenal Gland. As noted above, the adrenal gland appears to be most susceptible of the endocrine organs to chemically-induced structural change. Susceptibility appears to be due to the accumulation of lipophilic toxins in the lipid stores of the cortical cells and the metabolic conversion of chemicals to reactive toxic compounds (Capen 2001). In addition, such changes appear to be frequently associated with alterations in reproductive organ function and structure (Maronpot 1987). Even so, the range of histological responses in the adrenal gland is small (Tucker 1998). The most commonly induced morphological change is hypertrophy with increased vacuolar degeneration (Ribelin 1984). Cortical (interrenal) hypertrophy resulting from impaired steroidogenesis and the accumulation of precursors, such as neutral fats, in the cortical cells is found in animals treated with either endogenous or exogenous steroids. The hypertrophy can be so great that it impairs cellular function. Administration of adrenocorticotropic hormone (ACTH) will also cause hypertrophy of the cortex from increased size of the cells. In contrast, exogenous steroid treatment causing depressed ACTH levels results in atrophy of the cortex in mice. Cortical hyperplasia may also result from hormonal disruption, and propylthiouracil has been shown to cause proliferation in chromaffin cells medulla of mammals (Tucker 1998; Ribelin 1984).

Because of adhering fat, adrenal glands can be difficult to excise cleanly for organ weight measurements. To avoid variation in adrenal weights, a standardized necropsy protocol for the tissue should be established.

7.1.5 Japanese Quail Male Cloacal Gland Measures

The cloacal gland is a secondary sex characteristic of male Japanese quail that develops in response to testosterone and has been used to measure androgen status in the male during sexual maturation (Ottinger and Brinkley 1979a, 1979b). Typically, the length and width of the gland is measured with calipers, and the area is recorded as the product of longest length and greatest width of the gland. Coefficients of variation are typically low (4 to 10) for this measure. Measurement of just the length of the cloacal gland has also been used successfully to demonstrate the effect of hormone treatment on maleness in Japanese quail (Hutchison 1978) and cloacal gland volume in cubic centimeters has also been used to assess reproductive activity (CV of 4%) using the following formula

$$4/3\pi ab^2 \qquad (2)$$

where a is half of the length of the long axis and b is half of the length of the short axis (Chaturvedi et al. 1993).

Treatment with xenoestrogens *in ovo* has been shown to significantly decrease the area of the gland in the sexually mature male (Halldin et al. 1999), and cloacal gland swelling has been used as a reliable indicator of androgenic activity in females treated with testosterone (Phillips et al. 1997). Treatment of males with estrogen results in reduced gland area and deformities in the cloacal orifice (Phillips et al.1997; Halldin et al. 1999). As a measure that can be taken repeatedly during the course of a reproductive test, it is a useful endpoint for monitoring maturation and androgen status in P1 and F1 parent populations. The amount of foam in the cloacal glands is also used to determine male reproductive fitness. The cloacal gland is palpated and the proteinaceous foam measured with calipers and reported in units of square millimeters (Sachs 1967; Hutchison 1978). However, foam measurements can be highly variable. Date of actual foam formation is used as a maturation endpoint for the male Japanese quail. This measure is applicable to the P1 (if a prematuration exposure is used) and F1 chicks as they approach breeding.

7.2 Sexual Differentiation (Including Time of Onset of Egg-laying)

As described above in Section 7.1.2, *in ovo* exposure to estrogenic compounds will modify sex organ differentiation in birds, feminizing male sex organs and causing Müllerian duct abnormalities in genetic females. Failure of male gonadal differentiation in the presence of ecoestrogens may not persist from embryonic exposure into adulthood, but abnormalities are conserved in females (Rissman et al. 1984; Adkins-Regan et al. 1995). Ovotestes regress after hatch and disappear by sexual maturation (Scheib and Reyss-Brios 1979). Reorganization of the POM in the hypothalmus as a result of exposure to extrogenic compounds can result in apparently normal males that lack sexual behavioral differentiation (Berg et al. 1999).

Sexual differentiation endpoints include an array of morphological and behavioral landmarks that are easily recorded during the course of a reproductive toxicity test. The sequence of behavioral and morphological landmarks of sexual differentiation in male Japanese quail has been described (Ottinger and Brinkley 1979a, 1979b). Their onset and maximum expression are highly correlated in time with serum testosterone levels. Table 7-1 lists these landmarks in sequence of their appearance during sexual maturation. Also listed are estrogen-dependent landmarks that include appearance of dimorphic plumage and the onset of egg-laying in females. Age at sexual maturity was among the most sensitive measures of impaired reproduction in the female birds exposed to lead and was detected at dietary concentrations that caused no body weight loss or overt signs of toxicity (Edens et al. 1976). Onset of egg-laying was delayed by up to 2 weeks in lead-treated birds. In turn, peak egg production was also delayed and in most of the treatment groups. Similar delays in onset of egg-laying have been observed in female quail in a restricted feeding program (Zelenka et al. 1984). Timing of the transition from somatic growth to gonadal growth (sexual maturity) is influenced by accumulation of energy reserves in the juvenile stage. Therefore, chemicals such as OP pesticides that alter growth rate will influence the onset of sexual maturity, especially in the female (Zelenka et al. 1984).

7.3 Secondary Sex Characteristics

Development of secondary sexual characteristics in males and females are predominantly determined by differences in steroid secretion of the gonads of each sex. Perturbations in the development of these characteristics can affect the reproductive performance of the animal (Bortone et al. 1989; Bortone and Davis 1994). Because secondary sex characteristics in quail are hormonally controlled, they may be useful endpoints in endocrine disruption detection. Major secondary sex characteristics of Japanese quail include body size, distinctive plumage dimorphism, and the development of a foam-producing cloacal gland and sternotracheal (syringeal) muscles in males (Adkins 1975; Balthazart et al. 1983; Schumacher and Balthazart 1984). Formation of medualary bone in females generally begins 10 days before egg-laying under the influence of estrogen and testosterone, and could be considered a secondary sexual characteristic of female quail. It is readily induced in males by exposure to estrogens (Johnson 2000). Classic radiographic techniques or X-ray bone densiometry can be used to detect changes in medulary bone formation (Schreiweis et al. 2001). Initial equipment cost range from about $14,000 to $35,000 for digital formats. Some hand-held systems are available at lower cost that have had use in *in vivo* measurements of humerus and ulna radiographic density evalautions for poultry growth traits; however, these systems use radioisotopes with relatively short half-lives that must be replaced as often as every 6 months at $1300 per replacement, and image quality diminishes as the isotope decays.

In New World quail such as the bobwhite, plumage dichromatism is thyroid-estrogen based: that is, when the bird is adequately saturated with thyroid hormones and estrogen, it induces the development of female-like plumage. Lack of estrogen results in male phenotype (Hagelin and Ligon 2001). Low thyroid hormone concentrations result in asexual, juvenile-type feathers. In the past, it was generally thought that male plumage was under androgen control and was the primary criterion for female birds' mating decisions. However, it is now recognized that traits such as testosterone-mediated breeding displays and body size reflect male condition and are

used by females of some species to select mates (Hagelin and Ligon 2001). It is possible for very high testosterone exposure to result in female plumage in males due to the aromatization of testosterone to estrogen that would superimpose the estrogen-dependent female-type plumage on the male. Also, posthatch treatment of male Japanese quail with estradiol monobenzoate has been shown to result in plumage that resembled that of female Japanese quail (Hutchison 1978). Changes in plumage characteristics has been reported as number of individuals per group displaying spotted (female phenotype) or rufous colored (male phenotype) breast feathers. The width of the brown spots covering the breast region has also been used to quantify degree of feminization in males (Hutchison 1978).

The cloacal gland (Section 7.1.5) is a secondary sex characteristics that is used to determine maturation of male Japanese quail. Bobwhite males lack this gland. Maturation is determined by size and morphological features of the gland and by it secretory activity (Wada et al. 1992). The gland begins to enlarge as the bird approaches sexual maturation and takes on a red-colored appearance. A proteinaceous foam is also produced in response to circulating androgen and secretion of foam, but not fluid, is a sign of maturation. Foam quantity has also been measured as an additional correlated variable to plasma testosterone, but the measure is subject to collection variability. These endpoints have also been used to provide qualitative information that could be used to support evidence of endocrine disruption (Wada et al 1992; Halldin et al. 1999). Sternotracheal muscle weight (combined left and right muscles) has been used occasionally to evaluate the effect of estrogenic treatment in male Japanese quail (Hutchison 1978).

Most of the measures for these secondary sexual characteristics are relatively simple, though somewhat subjective, and can aid in interpretation of reproductive results.

7.4 Sex Ratio

Direct exposure of chicks and adults or material transfer of an endocrine-active compound and/or its transformation products to the egg can produce and inappropriate estrogen signal that can cause permanent sex reversal of behavior in male birds and transient feminization of gonadal tissue, if exposure is discontinued (Scheib and Reyss-Brios 1979; Berg et al. 1999). Female quail exposed to antiestrogens in *in ovo* display masculine behaviors as adults (Schlinger and Arnold 1995; Ottinger and Abdelnabi 1997). Sex ratios skewed toward a high number of females have been found in populations of birds exposed to estrogenic contaminants in the Great Lakes (Feyk and Giesy 1998). Although the ovotestis is usually discernable from a normal testis, at high doses of estrogenic contaminants, it can be difficult to distinguish an ovary from an ovotestis, and alternate method of sex determination will need to be used. Sex ratio should be determined for a representative group of the F1 and F2 14-day survivors. To assure that the chemical has had opportunity to transfer to egg, hatchlings from one of the later hatches, for example, at Week 4, should be genetically sexed at hatch. When the chicks reach 14 days of age, they should be necropsied, the presence of abnormal gonad recorded, and the relative amount of ovarian tissue determined *in ovo* testes for the males only. Gonadal weights obtained, presence of oviduct on right side, and the steroid status should be determined for all chicks.

Available laboratory techniques for determining sex include chromosomal (karyotyping) and DNA probe –that is, the Western Dot Blot and polymerase chain reaction (PCR) methods. Of these methods, DNA-sexing is preferred for monomorphic or immature birds. Karyotyping involves growing the sampled tissue, such as feather pulp, in culture for 7 to 9 days and microscopically identifying the Z and W chromosomes in a stained preparation based on morphological features. Commercial laboratories charge between $35 and $100 per sample. However, karyotyping is not considered to be as reliable as the new DNA probe methods. DNA probes are labeled DNA fragments cloned from chromosomes. DNA probes have recently been developed that can accurately distinguish the DNA in the sex chromosomes of male and female birds. In Western Dot Blot assays, each DNA sample is placed onto a nylon membrane using a dot blot apparatus. The membrane is then hybridized with a chemoluminescent, sex-specific DNA probe and exposed to an X-ray film to illuminate the differences between male and female samples. This method is very reliable when sufficient amounts of high-molecular-weight genomic DNA can be collected.

PCR methods use a single set of primers to simultaneously amplify homologous segments of the CHD-W and related Z-linked gene, CHD-Z (Griffiths and Kom 1997). Because the two CHD products are of the same size, a restriction enzyme is employed to cut a fragment of the CHD-Z product before gel electrophoresis (Ellegren 1996; Griffiths et al. 1996). This assay has been recently modified (Griffiths et al. 1998) to use two PCR primers that anneal to conserved regions of the gene and amplify across an intron that differs in length between in the CHD-W and CHD-Z genes (Khan et al. 1998). Thus, the PCR products are of different sizes, and a restriction enzyme step is not required to differentiate between the sexes. Both dot blot and PCR methods are extremely accurate and can make use of blood or feather samples. Because only a drop of blood is needed, claw-clipping will provide a sufficient sample if the blood spots are simply collected on filter paper. One feather, freshly plucked in a manner that obtains cells from the calamus, is an adequate sample (Taberlet and Bouvet 1991). Blood and feather samples for DNA-sexing require no special storage and can be archived for years. Experience is vital to perform these assays.

Costs at commercial laboratories for sexing birds using DNA probes range from about $17 to $20, and $22 to $25 per sample for blood and feather samples, respectively. Equipment cost to outfit a laboratory for dot blot assays can be as low as $2000 to $3000. For PCR methods, initial equipment cost can be $5000- $10,000 for manual methods and as much as $75,000-$100,000 for highly automated, high through-put fluorescent TaqMan analysis. If manual methods are

compared, 400 samples could be completed in one day by dot blot, but it would require 3 days to complete the same number by PCR.

7.5 Biochemical Measures

There are many hormonal actions of compounds that are categorized as EDCs. Endocrine disruption includes estrogenic, antiestrogenic, androgenic, and antiandrogenic effects, growth factor modulation, cytokine modulation, modulation of hormone metabolism, and many other activities (Jimenez 1997). The primary function of estrogenic substances is control of ovulation, and hence, reproduction. Secondary functions of estrogenic substances include gender determination, development of secondary sex characteristics, regulation of mating and breeding behaviors, and regulation of calcium and water homeostasis. The major differences between the effects of estrogen in mammals and egg-layers are the production of the egg yolk protein VTG in the liver, and eggshell formation in oviparous species. In birds, the primary sites of estrogen production are the gonads, but the brain also contains significant aromatase activity, suggesting that the brain is another major source of estrogen.

Estrogen production is regulated through a negative feedback loop by the pituitary peptide hormones, GnRH, leutinizing hormone (LH), and FSH, a system that has been studied in the Japanese quail primarily within the context of reproductive aging (reviewed by Ottinger et al. 1997). From the current understanding of reproductive biology, almost any hormone involved in the reproductive cycle is a potential biochemical marker for endocrine disruption. The transport of steroid hormones through the body, however, depends on the presence of various serum transporter proteins, which may differ across vertebrate classes. Steroid hormones readily pass through cell membranes and interact with receptors either in the cytosol or in the nucleus. Steroid hormones are metabolized primarily by the liver, where enzymes make them biologically inactive and water-soluble. The water-soluble conjugates are released into the blood and excreted in the urine or bile. Consequently, measures of liver enzyme activity or steroid byproducts in excrement are also potential biochemical measures of endocrine disruption. GnRh is currently being investigated as a potential endpoint for detecting endocrine disruption in avian reproduction toxicity tests by Mary Ann Ottinger and colleagues. To date, GnRH system appears to be robust, and therefore, the GnRH endpoint does not appear to be a sensitive indicator of endocrine effects.

Endocrine disruption is only one mechanism by which a chemical can interfere with reproduction and development. Thus, there is continued debate about whether endocrine disruption should be narrowly defined to pertain only to subcellular/cellular effects associated with the nuclear hormone receptor signaling pathway, or to be more inclusive of all aspects of production, release, transport, metabolism, binding action, and elimination. Others argue that endocrine activity of xenobiotic compounds is meaningful only if changes occur to the whole organism, and therefore testing should include methods for measuring effects at all levels of biological organization. In practice, the test methods to assess biochemical endpoints vary considerably, and include production of gene products, cell proliferation assays, tissue responses, VTG induction, hormone assays, egg production and fertility studies, and development of secondary sex characteristics (Jimenez 1997; Fairbrother 2000). Specific test methods that have

been performed on birds include subcellular measures of receptor binding, gene activation and protein; DNA interactions; tissue endpoints of hormone concentration or enzymatic activity; and whole-organisms studies with a focus on blood plasma concentrations of analyte (Fairbrother 2000).

7.5.1 Vitellogenin and Other Biomarkers of Hepatic Metabolic Changes

All oviparous species produce VTG, a protein absent in mammals. Both male and female livers may produce VTG, given sufficient estrogenic stimulation. VTG production is stimulated primarily by 17β-estradiol; estrone is only 5% to 10% as effective. VTG is released from the liver into the bloodstream, where it binds with free calcium ions, resulting in elevated total plasma calcium, as more ions are released from bone storage sites. Induction of vitellogenesis in Japanese quail leads to increases in the plasma concentrations of organically-bound phosphorus, bound calcium, and total protein (Robinson and Gibbins 1984). In female birds, oocytes take up the VTG and hydrolyze it to produce the yolk proteins, phosvitin, and lipovitellin. The synthesis of 1,25-hydroxycholecaciferal in the kidney is also affected indirectly by estrogen, where increased levels of 1,25-hydroxycholecaciferal increase absorption of calcium through the gastrointestinal tract. Exposure to environmental estrogens such as OC pesticides and phytoestrogens (Robinson and Gibbins 1984) induces this egg-forming system and is easily assessed in birds by measuring their VTG plasma levels. However, VTG is present in the plasma of female birds before ovulation. Therefore, it cannot be used as a biochemical marker of endocrine disruption in reproductively active females, because the natural production of VTG will obscure the contribution of EDCs.

The VTG structure varies among species and vertebrate classes. Wang and Williams (1980), for example, identified two distinct VTGs from white leghorn roosters based on amino acid, phosphorus composition, peptide maps, immunological reactivity, and relationship to the yolk lipovitellins. The authors concluded from these studies that the two proteins are distinct gene products that serve as precursors to different lipovitellin polypeptides. Similar findings were reported for the Japanese quail (Gibbins and Templeton 1982). These results have implications for the development and/or use of nucleic acid markers of endocrine disruption, such as messenger ribonucleic acid (mRNA), in that a mechanistic understanding of VTG production will be required to select the most appropriate mRNA target for study and detection. Heppell et al. (1995) attempted to develop a universal VTG assay by developing mono- and polyclonal antibodies against a conserved region of fish VTG sequences. The monoclonal antibodies did react with chicken plasma as measured by enzyme linked immunosorbent (ELISA) and Western Blot assays, but not with the same sensitivity or specificity as it did with the different fish species. More importantly, however, there was no conclusive evidence that the monoclonal antibodies specifically react to VTG in the plasma, especially in the chicken, and no data were shown relative to the reaction of the so-called universal polyclonal antibody with bird plasma. Thus, VTG assays continue to be species-specific and difficult to compare across species or vertebrate classes.

Current methods for detecting VTG levels in oviparous animals principally rely upon tissue endpoints and immunological techniques such as ELISA and radioimmunoassay (RIA).

Induction of vitellogenesis has been measured indirectly in quail by determining plasma levels of protein-bound phosphorus, total calcium, and total protein by conventional methods (Robinson and Gibbins 1984). Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) and rocket immunoelectrophoresis have also been described for quail plasma (Robinson and Gibbins 1984). VTG was successfully detected and quantified in plasma from male Japanese quail injected with estrogen analogs or zearlenone by the rocket electrophoretic technique, but the method was unable to detect VTG in male birds injected with the weakly estrogenic compounds o,p[1]-DDT, chlordecone, or methoxycholor. ELISA and RIA systems are based on the highly specific reversible reaction between an antigen–VTG, in this case–and a specific antibody. Of the two methods, RIA can be more sensitive because it does not require dilution of samples to reduce the interferences encountered in ELISA techniques. However, ELISA methods do not require the use of unstable reagents or radioisotopes and are easier to set up. Both ELISA and RIA assays are readily adapted to production as commercial kits or to routine use in commercial laboratories, once specific antibodies have been developed. Cost per sample for VTG analysis in fish by these methods range between $15 to $25.

As noted above, there is no universal VTG antibody available. Until very recently, the necessary specific and highly reactive antibody for quail VTG had not been produced, and no specific adaptation of either method for avian VTG is described in the current literature. However, Masaru Wada (College of Arts and Sciences, Tokyo Medial and Dental University) has recently developed a reportedly highly sensitive quail VTG ELISA assay kit in collaboration with Transgenic, Ltd., in Kumamoto Prefecture, Japan. The assay was developed using a rabbit anti-Japanese quail lipovitellin antibody and is being evaluated for cross-reactivity with other species. This method will be presented at the European Union SETAC 2002 Invited Symposium on Avian Endocrine Disruption. Wada has also developed an assay for circulating very low-density lipoprotein, another estrogen-sensitive yolk lipoprotein precursor in quail. Very low-density lipoprotein is separated from the plasma matrix in this method by using an automated lipoprotein assay system. It is then purified using high-pressure liquid chromatography, and is detected enzymatically. No data on the sensitivity and validation procedures for these assays are available at this time.

Tissue endpoints, such as VTG induction or enzyme activity measures, continue to be the preferred biochemical markers of endocrine disruption. As noted above, however, there is a lack of standard protocols and most assays require species-specific antibodies due to structural differences in the VTG peptides in different vertebrate classes. The VTG test alone may also necessarily miss other potential estrogenic effects, and the relative sensitivity of the VTG process compared with other estrogen-induced peptide and protein responses has not yet been established. Until such time, it should not be assumed that the VTG induction test would be a sensitive predictor of estrogenicity of xenobiotics in all oviparous species (Fairbrother 2000). Major limitations with *in vivo* assays are that they use highly complex responses that can be modulated through mechanisms that do not directly involve steroid receptors, and therefore are not necessarily selective for substances that act through these receptors. Nonetheless, *in vivo* studies are essential for examining endocrine activity, because they account for pharmacodynamic and pharmocokinetic interactions.

No *in vitro* assay for avian VTG has been developed, as exists for fish. However, avian hepatocytes have been cultured for use in chemical toxicity assays (Hugla et al. 1996; Kennedy et al. 1996) indicating that there is potential for the development of an *in vitro* screening assay for VTG in birds.

Not only is the liver the major site of VTG synthesis in birds, but it is also the site of many other enzymatic processes that directly or indirectly contribute to reproductive fitness. For example, birds that consume petroleum-contaminated foods develop an increased ability to metabolize the circulating contaminants through the action of a substrate-inducible mixed function oxidase system. Although the primary function of the system is to rid the organism of the contaminants, it may also accelerate the turnover of some endogenous substrates, such as steroid hormones. Indeed, studies conducted with halogenated aromatic hydrocarbons (HAHs) have shown a link between HAH-exposure, liver enzyme function and health effects, such as developmental toxicity, hepatotoxicity, endocrine disruption, immunotoxicity, and death. Although the exact role of cytochrome P450 in HAH toxicity is not clear, both the toxic and biochemical responses, such as cytochrome P450-induction, appear to be mediated by a shared mechanism that involves changes in gene expression initiated by binding of HAH to the aryl hydrocarbon receptor. Cytochrome P450-induction in cultured cells therefore serves as a sensitive marker of the activation of aryl hydrocarbon receptor-dependent pathways, and measures of liver enzyme activity could be useful indicators of endocrine disruption.

Liver enzyme activities in birds have been studied most thoroughly with respect to HAH and polychlorinated biphenyl (PCB) exposures. The principal biomarker continues to be cytochrome P450 1A-induction, assessed by measuring 7-ethoxyresorufin-O-deethylase (EROD) activity using 7-ethoxy resorufin as substrate and a fluorescent plate reader. Cytochrome P450 1A concentration is typically quantified via immunological techniques, such as RIA, ELISA, or Western Blot. Kennedy et al. (1996), for example, determined concentration-dependent effects of HAHs in primary hepatocyte cultures prepared from the embryos of four breeds of chicken, pheasants, turkeys, three breeds of duck, and herring gulls. Results from this study, and comparison with prior *in vivo* or *in ovo* studies, suggest that it could be possible to predict the sensitivity of a species to *in ovo* lethality by HAHs from the relative sensitivity of hepatocyte cultures to EROD-induction. A practical implication of this research is that hepatocyte cultures could be used to estimate the sensitivity of rare or endangered wild bird species.

Brunstrom and Halldin (1998) also assessed EROD-induction in chicken embryo livers to examine whether there are similar interspecific differences in the EROD-inducing potencies of aryl hydrocarbon receptor agonists compared with embryo toxicities. The EROD assay was also extended to hen, turkey, domestic duck, Japanese quail, eider duck, and common tern eggs. The authors found good agreement between EROD-inducing potency and embryo toxicity, measured as lethality or malformations, which supports the notion that species differences in EROD-induction reflect differences in an aryl hydrocarbon-receptor-mediated enhancement of Cytochrome P450 1A levels. The authors note, however, that differences in chemical injection method, carrier solution, and embryo age are known to affect the extent of EROD-induction or activity. Further, the relative potencies of HAHs or PCBs can vary considerably among different

avian species; the chicken appears to be the most sensitive of the birds. Freeman and McNabb (1991) pointed out that many enzyme activity assays have never been validated to specifically measure initial velocity conditions, such that many published estimates of enzyme activity could actually underestimate the true enzymatic activity of the cells or tissue. Assay standardization and validation are therefore prerequisite to the use of enzyme activity as a biomarker for endocrine disruption.

Other enzyme activities measured in hepatic tissues or cell cultures include naphthalene-metabolizing activity in mallard ducks, using a radio-labeled (I^{125}) substrate to measure specific activity as nanomoles naphthalene/minute/milligram microsomal protein (Gorsline and Holmes 1981). A 5'-deiodinase activity has been used as a biomarker in the Japanese quail; it is an assay that is based on initial velocity conditions (Freeman and McNabb 1991). There, initial velocity conditions were defined as those where the enzyme activity increases proportionally with increasing enzyme concentration and increases linearly and proportionally with increasing incubation time. In this study, the authors measured the release of I^{125} from rT3 in liver homogenate and several other developing tissues from 12 h to 2 days of age. Of particular import to other enzyme activity measures was a demonstration that different sets of assay conditions could be selected for making quantitative measurements under initial velocity conditions, and that a validated assay can then be used to compare the effects of different endocrine disruptor treatments.

The estrogen-synthesizing enzyme aromatase, a member of the cytochrome P450 family, has been colocalized with estrogen receptors in the brain of Japanese quail (Dellovade et al. 1995) by immunocytochemical techniques and has been assayed in other contexts (Balthazart et al. 1996; George and Wilson 1982; Schlinger and Arnold 1992), but not specifically as a biochemical measure of endocrine disruption. In birds, the enzyme plays an important role in determining sexual characteristics and reproductive behavior (Elbrechet and Smith 1992; Schlinger and Arnold 1992). Ottinger and coworkers at the University of Maryland are currently conducting a preliminary application of this technique in a one-generation reproductive test using northern bobwhite and Japanese quail challenged with methoxychlor, a weak estrogenic compound. If it proves to be a sensitive indicator of endocrine disruption in the various life stages of the birds, modifications to the histoimmunochemical technique to convert it to an ELISA would be useful to provide a more routine procedure for endpoint detection.

Although nucleic acid assays are typically conducted within the framework of deducing metabolic pathways and mechanisms, measuring mRNA- or DNA-protein interactions can also be used to assess hepatic metabolic changes. For example, Gordon et al. (1988) studied the effects of estrogen on the stability of mRNAs that code for the yolk precursor proteins apolipoprotein and VTG II in white leghorn cockerels. Total RNA was isolated from liver tissue and used for Northern Dot Blot analyses. Solution-hybridization assays were also performed to measure mRNA decay constants. Gupta and Kanungo (1996) used total RNA isolated from the liver for Northern Blots and gel mobility shift to study the transcriptional regulation of the VTG operon. Edinger et al. (1997) used nucleic acid analysis to address the concept of hepatic memory in chickens, a concept that stems from observations that after the initial activation of the

egg yolk protein genes by estrogen, subsequent responses to estrogen occur more rapidly. In this paper, the enzyme deoxyribonuclease (DNAse)-I footprinting was performed to identify DNA: protein complexes in the estrogen response element. Run-on transcription assays were also performed to determine the extent of or delay in accumulation of apolipoprotein mRNA during transcription. Shimada et al. (1996) used Northern Blot and slot blots to study the induction of cytochrome P450 mRNA. Although the statistical power of nucleic acid techniques such as these to interrogate metabolic changes in mRNA production is widely known in the literature, they have yet to be applied as endpoint measures of endocrine disruption.

7.5.2. Plasma and Fecal/Urate Hormone Concentrations; Estrogen, Testosterone, GnRH, T3/T4, TSH, Corticosterone

The hepatic enzyme measurements described above require test animals to be sacrificed, a practical consideration that is not conducive to profiling changes in endocrine status in test animals. Thus, assays have been developed to measure many of the hormones and proteins in blood plasma and urine. With the advent of RIA systems in the 1960s and 1970s, circulating hormones could be easily and accurately quantified. For example, in a study with quail, the minimum detectable dose of estrogen was 3.8 pg /tube, the concentration at 50% binding was 188 pg/mL, and the interassay CV was 7.6% (Soh and Koga 1994). Androgen assay by single antibody RIA kit has been validated for use in Japanese quail plasma by Ottinger and Mahlke (1984). Intra-assay CV was 5%. Corticosterone concentrations were measured by a double antibody RIA by the same authors. Originally developed to measure the circulating titers of sex steroid hormones in mammals, RIA assays were readily adapted for use in birds because of the similarity of steroid hormones among phyla (Wingfield and Farner 1975). Serum thyroid hormones have also been quantified in Japanese quail treated with endocrine-active compounds, such as DDT, PCBs, and thiouracil, by RIA using commercial mammalian kits (e.g., Grässle and Biessmann 1982). RIA methods for gonadotropins are sufficiently accurate to detect changes of 20% in mean blood hormone levels with group sizes of 20 or more (Thorell and Larson 1978). RIA continues to be the main technique for hormonal analysis in plasma and other sample sources (Ottinger et al. 2001; Jones and Satterlee 1996; Watson et al. 1990; Jones et al. 1992; Adkins-Regan et al. 1995; Schlinger and Arnold 1992; Wingfield and Farner 1975; Nisbet et al. 1999; Millam et al. 1998; Abdelnabi et al. 2000; Marai et al. 2000; Bluhm et al. 1984) due in part to the ease with which specific antibodies can be developed, and the sensitivity of radioactive assays. However, fluorescent immunoassays such as ELISA are becoming more prevalent, thus eliminating the hazards of radiolabeled substrates (Tell and Lasley 1991; Millam et al. 1998; Alston-Mills et al. 1989; Otani et al. 1993). Despite the number of studies on circulating hormones in birds, there are relatively few studies concerning the relationship between circulating hormone levels and endocrine disruption. Gorsline and Holmes (1982) investigated the mechanism whereby petroleum contaminants alter adrenocortical function by studying the distribution, metabolic clearance rate, and estimated *in vivo* secretory rate of corticosterone in mallard ducks. Results showed that lowering of the plasma corticosterone concentration in petroleum exposed birds is associated with a decline in corticosterone secretory rate. Biessmann (1982) studied the effects and mode of action of PCBs on gonads and sex hormone balance in juvenile quail during sexual maturation by measuring plasma concentrations of 17β-estradiol, testosterone, 5-α-dihydrotestosterone, and progesterone. 17β-estradiol and

calcium were unaffected by PCB. Cavanaugh and Holmes (1982) studied plasma estrogen and progesterone concentrations to determine whether impaired reproductive measures in mallard ducks exposed to petroleum are due to an endocrine dysfunction in the ovary. None of their present evidence suggested that circulating petroleum hydrocarbons can stimulate estrogenic change by binding to estrogen receptors. Some hydrocarbons seem to impair reproductive cyclicity in a manner suggesting impairment of gonadal steroid hormone synthesis. The authors suggest this second type of effect was probably responsible for the disturbances observed during the study. A subsequent study (Cavanaugh and Holmes 1987) suggested a high probability that petroleum products accumulate preferentially in steroidogenic cells in the developing ovaries, such that petroleum compounds can interfere directly with gonadotropin-dependent steroid hormone synthesis.

Unfortunately, frightening or painful stimuli can cause instantaneous changes in endocrine systems, particularly the hypothalamic-pituitary-adrenal axis. Disturbances such as movement of the cage or handling of the animal can alter some circulating steroid concentrations. Therefore, blood sampling methods that subject the animal to minimal stress will result in more accurate concentrations of hormone concentrations in the circulation. Application of fecal/urate sampling to reproduction toxicity testing in birds is a means of sampling for steroids with minimal disturbance to the birds.

Measures of the steroid or steroid metabolite content of feces have been used in conservation biology for over a decade. These measures have been shown to reliably detect hormone status and adrenal activity in a wide array of mammalian species (Wasser et al. 2000) and avian species (Tell and Lasley 1991; Wasser et al. 1997). It is currently being adapted for use in monitoring endocrine changes in birds (Brewer et al. 2002a, 2002b; J. Clark, A. Faribrother, L. Brewer and R. Bennett, personal communication, 2002, Effects of exogenous estrogen on mate selection by female house finches [*Carpodacus mexicanus*], submitted paper). The method involves collecting fecal samples, or fecal/urate samples in the case of birds, extracting the steroid or metabolites, and analyzing the extract by RIA. Extraction methods, originally involving labor-intensive sequences of extraction and chromatography steps, have been replaced by a rapid method validated by Wasser et al. (1991). Current fecal extraction methods are based on modifications by Brown et al. (1994) and Wasser et al. (1994). The estrogen and testosterone content of the extracts are analyzed by double-antibody I^{125} RIA using commercially available kits. Recovery of 17β-estradiol from spiked fecal samples has been reported at 99.2% ± 9.1%. Assay sensitivity for estrogen was 1.25 pg/tube, and the interassay CV was reported to be 6.7%. In the testosterone assays, recovery of testosterone was also 99.2% ± 9.1%, the assay sensitivity was 0.1 ng/mL and the interassay CV was 11.1% to 14.2% (Wasser et al. 1991). Similar recovery and CV are obtained for progesterone analyzed by single-antibody I^{125} RIA that cross-reacts with progesterone metabolites (Brown et al. 1994; Wasser et al. 1994). The fecal steroid values are comparable to serum steroid secretory profiles measured in a number of wild species (Wasser et al. 1991, 1995, 2000). Development of an assay to measure glucocorticoid stress hormone in fecal material has only recently been developed. Assays for these hormones were difficult to develop because they undergo extensive and rapid metabolism prior to excretion that is both species-specific and subject to further transformation by gut flora (Erikson 1971; Palme et al. 1997; Bahr et al. 2000). Using a commercial I^{125} corticosterone RIA assay (ICN

Pharmaceuticals, Costa Mesa, California), Wasser et al. (2000) directly compared serum and fecal concentrations of corticosterone in the spotted owl (*Strix occidentalis caurina*) and showed that the metabolites measured in the feces accurately reflected the adrenal stress response induced by a natural stress (removal to a new location) and challenge by ACTH. The interassay CV was 5.5% for a high sample (27% bound) and 3.0% for a low sample (62% bound). Intra-assay CV was 5.18% for 30 pairs.

Because fecal/urate measures of steroid hormones are noninvasive, they can provide both more cost-effective methods for determining hormone status in test birds and much-needed endpoints that can be correlated to field investigations.[2] Sampling blood is an invasive procedure that involves capture and withdrawal of blood; because it stresses the birds, it should be avoided during egg-laying. This limits sampling for steroid hormones to prematuration periods and the termination of the study. Sampling of blood, as noted above, requires a large expenditure of labor and can be difficult to accomplish within an appropriate period of a circadian cycle. With fecal sampling, samples can be collected over the entire exposure period with minimal effort. Typically, validation involves direct comparison between blood and fecal levels, and the hormones of interest over one or more reproductive cycles for reproductive steroids, and ACTH challenge studies for glucocorticoids. A study correlating fecal/urate steroid concentrations with house finch nesting behavior was recently completed, and a second study comparing excreted steroid concentrations with plasma levels during the reproductive cycle of Japanese quail is currently in progress under funding from the American Chemistry Council (L. Brewer, personal communication, 2002). An indirect validation for life cycle studies is to track fecal hormone concentrations over time in the species of concern, comparing the monitored values with expected biologically relevant profiles. An evaluation of the effect on assay results of potential interferences such as phytoestrogens that may be present in diet and in excrement is needed. Cost per sample from noncommercial laboratories (e.g., university laboratories) in the U.S. range from $10 to $15 up to about $25. Fecal steroid analysis was extensively researched at San Diego Zoo as a noninvasive means of sexing monomorphic avian species, but as of yet is not currently commercially available.

The use of feces for noninvasive monitoring of thyroid hormones has not been reported; however, there is a high probability that similar assays can be adapted to these hormones because they are primarily excreted through the bile. There is growing interest among conservation biologist to develop a thyroid assay from feces owing to the importance of thyroid hormones as an index of starvation (S. Wasser, personal communication, 2002).

Regardless of the assay method or sample matrix, thyroid measures should be viewed with caution. Plasma T3 levels are of doubtful value for diagnosing hypothyroidism because of a common syndrome that evolves in animals experiencing various nonthyroidal illnesses, stress, or starvation. This syndrome, called Sick Euthyroid Syndrome, or Low-T3 Syndrome, could develop to protect the body from catabolic processes that accompany these conditions (Davison et al. 1985). Although T4 would then appear to be the better choice for evaluating

[2] Field preservation techniques have been developed.

hypothyroidism in birds, even plasma T4 concentrations can be influenced by handling, bleeding (Williamson and Davison 1985a), food intake (Williamson and Davison 1985b), and increased corticosterone concentration (Davison et al. 1985). Because of the short half-life of T3 and T4 in avian blood, plasma fluctuations of T3 and T4 render it difficult to document hypo- or hyperthyroidism from a single sample (Lumeij 1994; Oglesbee et al. 1997), such as would be taken at necropsy. Here the ability to monitor thyroid hormones over a period of time via sampling the feces could enhance the interpretive power of thyroid assays.

Whereas these prior studies demonstrate that circulating hormones and peptides are readily measured in birds, the obvious lack of mechanistic understanding currently precludes their effective use as biochemical markers of endocrine disruption. Current methods for assessing wildlife health effects are generally targeted at detecting effects rather than mechanisms, and may not adequately evaluate effects on the endocrine system. This is particularly true for exposures that occur during critical developmental periods (Jimenez 1997). In this regard, previously highlighted methods and approaches for assessing endocrine disruption must be considered within the mechanistic black box that defines risk assessment. For estrogen or an estrogenic substance to exert its effects, for example, it must bind to an estrogen receptor with sufficient affinity and specificity to elicit the downstream production of species-appropriate proteins. Thus, similarities among species hormones or estrogen receptor structures could be important in determining whether or not a chemical elicits an estrogenic or antiestrogenic response. Further, receptor binding assays are limited because they only measure the ability of a chemical to bind to the estrogen receptor and do not include any measure of its ability to pass through the cell membranes and contact the nuclear receptors, nor do they reveal whether the receptor binding initiates mRNA transcription. Receptor binding assays also suffer from limitations imposed by the artificial situation whereby substances are tested for their ability to compete with small amounts of radiolabeled tracer estrogen for binding sites. This is different from the *in vivo* situation, where substances compete with greater amounts of natural hormones that generally have a much higher affinity for the estrogen receptor (Fairbrother 2000).

8.0 RESPONSE TO ESTROGEN AGONISTS AND ANTAGONISTS

The potential for environmental chemicals to mimic the effects of estrogen in birds was first reviewed by Rattner et al. (1984). The potential for such interactions to result in feminization of males (both in wildlife and in humans) focused attention on the endocrine disrupting mode of action of reproductive toxicants (Colborn et al. 1996.). As a result, a large amount of work has been done in the past decade to develop sensitive assays for detection of estrogenic effects of xenobiotics, mostly focusing on mammalian systems, but more recently assessing applications to

fish and birds (Ankley et al. 1998; DiGuilio and Tillett 1999), and invertebrates (DeFur 1999). Initial concerns focused on estrogen mimics, primarily due to binding to the estrogen receptor and stimulating down-stream responses; however, scant attention has been paid to detection of antiestrogenic effects of chemicals, such as receptor blockers.

8.1 Sexually Mature Life Stages

All vertebrate classes share some similarities of response to estrogenic stimulation, whereas certain aspects of avian physiology respond differently (Fairbrother 2000). Common responses are development and expression of secondary sex characteristics, reproductive behaviors, control of follicular growth and maturation, and calcium regulation. Adult oviparous species, such as birds, herpetofauna, and many fishes, also require estrogen for VTG synthesis, oviduct development and maturation, and shell gland function. Estrogen may regulate seasonal reproductive cycles through stimulation of the pineal gland to produce melatonin.

8.1.1 Sensitivity to 17β-estradiol or Synthetic Estrogen Exposure

Estrogen increases production of VTG by increasing the number of copies of VTG mRNA in hepatocytes. VTG production can be under the control of one gene, for example in the chicken, or of more than one gene, as in quail. Alternatively, different forms of vitellogenic peptides may result from posttranslational processes. Differential effects of estrogen on species with the various production modes have not yet been determined. VTG production is stimulated primarily by 17β-estradiol (see Section 7.5.1); potency of synthetic estrogens, such as diethylstilbesterol, on VTG production varies, but generally is less than that of estradiol. Estrogen also plays a role in passerine male singing behavior and on copulatory behaviors of both sexes (reviewed by Fairbrother 2000). Distinct estrogen-receptive neurons have been located in the brain, using [H^3]17β-estradiol.

In general, neuroanatomimical distribution in the brain of the steroid-receptive cells is similar across species. Enstrom et al. (1997) conducted a study of mate choice among dark-eyed juncos (*Junco hyemalis*), in which estradiol levels were manipulated. Treatment with estradiol enhanced female sexual behavior and promoted precopulatory displays, although estradiol-treated females had significantly smaller ovaries than control females.

Turner and Eliel (1978) studied the ability of DDT and its metabolites (o,p'-DDT, p,p'-DDT, o,p'-DDE, and o,p'-DDD) to compete with H^3-estradiol for binding to the estrogen receptor on Japanese quail oviduct cells. Only o,p'-DDT competed significantly for binding sites, but a large molar excess (20,000 X) was required. This suggests that o,p'-DDT has a very weak affinity for the estrogen receptor and likely will not elicit a true hormone-specific response in these cells.

Another chlorinated insecticide, chlordecone, also known as kepone, has been shown to have estrogenic activity at doses lower than that which causes systemic toxicity (McFarland and Lacy 1969). The greatest effect occurs when the pituitary is intact, because chlordecone stimulates the

hypothalamic-pituitary axis to release FSH. It also is possible that the pituitary trophic hormones increased the responsiveness of the oviduct to direct action by the chemical. Chlordecone also may block the release of LH, and prolong the release of FSH and ovarian estrogen secretion. It binds to nuclear estrogen receptors in the magnum and shell gland regions of the oviduct, and mimics estrogen effects in these locations, although its potency is only about 1/1000 of that of 17β-estradiol (Rattner et al. 1984). In males, depressed cloacal gland activity following chlordecone exposure could be due to inadequate LH release and associated depressed testosterone secretion.

8.1.2 Antiestrogens

There are no reports in the literature of effects of avian exposures to antiestrogenic chemicals. Pharmaceuticals such as raloxofene, tamoxifen, and danazol have been developed for use in women. Raoloxofene and tomoxofen are selective antagonists blocking the estrogen receptors on the uterus and breast tissue, but are agonists in other tissues, such as those in bone. Danazol also binds to and blocks numerous steroid receptors, including those for estrogen and testosterone, but its primary use is through the inhibition of production of FSH and LH by the pituitary. Tamoxifen has been used as an antagonist in estrogen research in poultry and exotic bird medicine (Lupu 2000), in which it is effective in controlling egg-laying. Recent investigations in sex-specific neural development have shown that tamoxifen is effective in inhibiting song circuit development in the male zebra finch (Holloway and Clayton 2001).

8.1.3 Relevance, Sensitivity, Use History, Uncertainty

Study of the potential for excess estrogen to affect behavior and reproduction of adult birds has been conducted primarily though the administration of the endogenous hormone 17β-estradiol. Although this regime definitely mimics the natural action of estrogen, it has been criticized by some because its efficacy could be affected by endogenous negative feedback cycles. Further, it does not provide a means of determining the consequences of effects on synthetic or degradative or metabolic pathways, such as cytochrome P450 systems, for estrogen. Use of synthetic estrogens such as ethinyletradiol (EE_2), which is a widely used contraceptive, or DES also provides a reasonable model in the avian system, but is susceptible to the same shortcomings as use of 17β-estradiol. There is a lack of suitable chemicals to serve as known antiestrogenic controls for bird studies. However, measurement of hormonally-driven behaviors such as singing, copulatory receptivity, and nest building, and concentrations of circulating or excreted hormones such as estradiol and estrone will provided the necessary information for determination of general mode of action of reproductively active chemicals.

8.2 Juvenile Life Stages

8.2.1 Sensitivity to 17β-estradiol or Synthetic Estrogen Exposure

Sexual dimorphism in terms of behavior and neuroendocrinology has been well studied in the Japanese quail. Female quail are demasculinized by endogenous estrogens (Hutchison 1978),

and embryonic treatment with estrogen during the critical period of brain development at Days 4-12 of embryogenesis results in an irreversibly depressed response of copulatory behaviors in the adult male to the activating effects of testosterone (Halldin et al. 1999). Likewise, estrogen suppresses development of the singing center in brains of embryonic passerines.

Berg et al. (1999) clearly showed that the synthetic estrogens EE_2 and DES exhibit significant estrogenic activity during embryonic maturation of Japanese quail, resulting in feminization of male embryos and malformation of the Müellerian ducts in females. EE_2 was 3 to 10 times more potent than DES, as indicated by a dose-dependent increase in the frequency of male embryos exhibiting an ovotestis. Halldin et al. (1999) then studied the effects of embryonic exposure to these same synthetic estrogens on sexual behavior in adult Japanese quail. Sexual behaviors were significantly depressed following *in ovo* treatment by either estrogen. Testis weight asymmetry was significantly increased by EE_2 but not by DES. The cloacal gland area was significantly reduced by treatment with DES. However, plasma testosterone concentrations did not differ significantly from the control values and were not correlated with frequency of sexual behaviors. This suggests that the *in ovo* exposure resulted in altered sensitivity of the brain to the activating effects of testosterone, rather than affecting the ability of the testes to produce the hormone. Taken together, these two studies indicate that EE_2 is more potent than DES during embryonic exposures.

Schumaker et al. (1989) identified Day 9 of incubation to be the time when embryonic development of Japanese quail is most sensitive to estrogenic effects. Estradiol benzoate (EB) treatment demasculinized sexual behavior and cloacal gland growth of males. However, some dependent variables, such as plasma levels of luteinizing hormone and crowing, were still affected by EB treatment on Day 14. A 5 μg dose of EB totally suppressed the capacity of the adult male to show copulatory behavior in response to testosterone, if it was present in the egg on Day 9 of incubation.

Treatment of embryonic Japanese quail and chickens by egg injection on Day 3 of incubation with the plastic monomere, bisphenol A, resulted in malformed Müllerian ducts at 2 days prior to hatch in female quail and ovotestes feminization in male chickens (Berg et al. 2001). Effective doses of bisphenol A were embryolethal in chickens, but not in quail. DES used as a positive control showed the same species-specific effects on Müllerian duct and ovotestes formation. DES appears to be five times more potent than bisphenol A in the quail, but only three times more potent in the chicken. Thus, target organs, gender effects, and sensitivity to xenobiotics appear to be species-specific. The flame-retardant, tetrabromobisphenol A, had no sublethal effects on the developing embryo of either species. Both of these compounds are readily excreted in the bile, and the authors acknowledged that it is possible that female birds do not deposit sufficiently high levels in the egg to result in the same effects observed in the laboratory egg injection studies.

Sexual differentiation is not a unitary process in quail: various responses differentiate at embryonic ages between Days 9 and 12, and require different levels of estrogen. The process

also is progressive, and some aspects of behavior and morphology are not complete until the posthatching period. Yoshimura et al. (2000) documented changes in gonadal growth and histology, age of sexual maturation, and ovarian weights of Japanese quail exposed to DES during the posthatch period , Day 0 to Day 53. Age of sexual maturation was delayed in males as a result of slower testicular development. Semen production and copulation activity appeared to be unaffected, as measured by egg fertility rates. In females, ovarian development also was delayed during early posthatch lifestages, although the age of sexual maturation was no different from controls. A greater number of ovarian interstitial cells were found in DES-treated birds than in controls. The authors suggest that these effects may be due to binding of DES to the estrogen receptor, stimulating downstream activities.

Feminization of male gull embryos occurred following injections of DDT at concentrations similar to those found in gull eggs from southern California (cited in Halldin et al. 1999), suggesting that environmental contaminants may have similar effects on embryonic development as the estrogens reviewed above. However, relative potency of the various chemicals is not known.

8.2.2 Antiestrogens

Very little is known about antiestrogenic properties of xenobiotics administered to birds. McMurry and Dickerson (2001) and others showed that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is both antiestrogenic and antiandrogenic in northern bobwhite quail. TCDD is approximately as potent as EE_2 and decreases eggshell gland weight in a dose-response fashion when injected into eggs prior to the start of incubation. Indole-3-carbinol is a naturally occurring, phytoantiestrogen, although it causes minimal effects–decreased weight gain–in developing bobwhite embryos (McMurry and Dickerson 2001). The result of the inhibition of aromatase in 5-day-old chicken embryos by 5-(p-cyanophenyl)- 5,6,7,8-tetrahydroimidaxo [1,5-α]pyridine hydrochloride was that all hatchlings developed as phenotypic males (Elbrecht and Smith 1992). Sex-reversed females developed bilateral testes that were capable of complete spermatogenesis and had the physical appearance and behavior of normal males. Similar effects occurred after exposure to ergosterol biosynthesis-inhibiting fungicides that also inhibit aromatase P450 enzymes (Dawson 2000).

8.2.3 Relevance, Sensitivity, Use History, Uncertainty

In birds, the developing embryo is the most sensitive life-stage to the activity of estrogens or their antagonists. Changes that occur during either *in ovo* or posthatch exposure will persist throughout the life of the bird and influence adult reproduction, even if exposure occurs only in the early life stages. Because birds develop in hard-shelled eggs, they are most likely to be exposed to such substances throughout embryogenesis, including the most sensitive stage of development, which is Day 9 in the quail. Some xenobiotics–OC pesticides, for example–require metabolic degradation into secondary metabolites prior to exerting estrogenic effects. It is not clear whether such degradation can occur in the egg, or whether the secondary products must be

deposited by the hen during egg formation. Regardless, once present in the egg, these products cannot be excreted as readily as they are in mammals, even though clearance of metabolic wastes by the bile does occur. Therefore, they may continue to pose a risk throughout the incubation period. Hatchlings can continue to be exposed for several days immediately posthatch, because they rely on the resorption of the yolk sac as an energy source during that time. It is clear that sexual development continues during the posthatch period and can be significantly influenced by increased levels of estrogen during that time. Both the natural and synthetic estrogens are reasonable models for use in demonstrating estrogenic effects during these life-stages. Further studies of estrogenic xenobiotics that require metabolic activation will determine whether or not this occurs in the embryo, and if so, its relationship to the sensitive developmental period.

9.0 RESPONSE TO ANDROGEN AGONISTS AND ANTAGONISTS

Birds appear to be affected by androgenic or antiandrogenic substances to a much lesser extent than they are affected by estrogens and antiestrogens. Because males are the homozygous sex in birds, sexual dimorphism results from a demasculinization of female embryos under the influence of estrogen; lack of estrogen results in production of phenotypic males (see Section 3.2). In contrast, excess or insufficient testosterone is unlikely to affect female birds, unless levels are so low that there is no substrate for the action of aromatase, and therefore no production of estrogens. Adult male and female birds have equivalent levels of circulating plasma testosterone. It has been shown that 17β-estradiol administered to the adult male quail induces receptivity, but females treated with testosterone fail to show male sexual behaviors (reviewed by Balthazart et al. 1983). Consequently, less research has been focused on understanding potential effects on testosterone-active xenobiotics in birds than on estrogen-related substances.

9.1 Sexually Mature Life Stages

9.1.1 Sensitivity to Androgenic Steroid Exposure

Testosterone exerts its influence through binding to cell receptors, entering the target cell, and then undergoing conversion into a number of metabolites. These metabolites bind to nuclear receptors and stimulate DNA to produce appropriate physiological responses. For example, the 5α-reduced metabolites (5α-dihydrotestosterone [5αDHT] and 5α-androstane-3α,17β-estradiol [5α,3α-diol]) activate strutting, crowing, and cloacal gland development. The production of the 5α-reduced metabolites is much higher in male cloacal glands than in females (Balthazart et al. 1983). However, in the brain of birds, a specific enzyme, 5β-reductase, reduces testosterone to 5β-dihyrotestosterone (5β-DHT) which then is converted to 5β-androstane-3α,17 -estradiol(5B,3α-diol). These β-metabolites are inactive; it has been shown that injections of 5β-DHT do not activate sexual behavior or development of secondary sex characteristics. The pituitary gland and syringeal muscles used in singing of females produced more of the 5β-reduced metabolites than was generated in these locations by males (Balthazart et al. 1983). Because circulating plasma levels of testosterone do not differ significantly between males and females in photostimulated Japanese quail, it is likely that sexual dimorphism is a result of the

differences in metabolic activation/deactivation reactions rather than in the capacity of ovarian or testicular tissues to produce testosterone (Balthazart et al. 1983).

Adkins-Regan and Ottinger (1988) demonstrated a pronounced diurnal rhythm in rate of clearance of injection-induced elevations in plasma testosterone levels in Japanese quail, with faster clearance rates occurring during the day than at night. In addition, many birds follow a cranial cycle as well. They are seasonal breeders, responding to increasing length of photostimulation by enlargement of the gonads and onset of copulatory behaviors. Some birds molt just prior to onset of reproductive capacity, and most begin their molt shortly after completing the breeding and/or brood-rearing phase of the reproductive cycle. At the onset of short day lengths, gonads are receptive to the inhibitory effects of reduced amount of light. Eventually, the gonads become insensitive to the gonadosuppressive effect of the short photoperiod, and reproductive activity increases. The onset and length of the period of sensitivity of the gonads to photosuppression varies by species. Treatment of day-old Japanese quail chicks with high levels of testosterone proprionate resulted in the development of testes that were always sensitive to the effects of short day length, and therefore never were stimulated to return to breeding condition. The same testosterone treatment in female chicks resulted in suppressed ovarian development.

Stoehr and Hill (2001) addressed the question of interference of hormones on molting and plumage coloration. They found that male birds exposed to exogenous testosterone close to their molt were likely to molt into plumage that was duller than that which they previously displayed. Males that had been treated with testosterone earlier in the year did not show as marked an effect.

9.1.2 Antiandrogens

Inhibitors of the enzyme that reduces testosterone to its active 5α-metabolites have been used as testosterone antagonists for the treatment of prostate cancer. However, it is not clear whether inhibitors of 5β-reductase, such as finasteride, will have any physiological effect on birds. The level of circulating testosterone will increase, at least transiently, which may have an effect on the amount of testosterone that is changed to the 5α-metabolites. In humans, finasteride is marketed under the name Proscar by Merck & Co., Inc., for the treatment of enlarged prostates; it shrinks the size of the organ. Treatment with the 5β-reduced metabolites does not result in any antiandrogenic effects.

Cyproterone acetate (CyA) is an antiandrogenic substance that has been shown to suppress nest-soliciting behavior and ovarian development of ring doves (*Columba palumbus*), copulatory behavior and external morphology of Japanese quail, and courtship behavior of zebra finches (*Taeniopygia guttata*) (Suresh and Chaturvedi 1987). The interaction of CyA with lengthening photoperiod, which is a reproductive stimulator, in the red headed bunting (*Emberiza bruniceps*) was studied by Suresh and Chaturvedi (1987). Under normal day lengths, CyA inhibited testicular growth and reduced body weight. It partially arrested the activity of the developing gonad during the photostimulatory period of lengthening days, although these effects were

completely and quickly reversed at the cessation of treatment. Effects of CyA were partially offset by simultaneous treatment with testosterone proprionate.

Another pesticide, DDT, and its degradative p,p'-metabolites, are weakly antiandrogenic, and TCDD is both antiandrogenic and antiestrogenic in northern bobwhite quail, depending upon dose and tissue (McMurray and Dickerson 2001). Neither of these OC compounds influences the levels of circulating hormone. Their mode of action is similar to that of vinclozolin, acting as androgen receptor antagonists.

9.1.3 Relevance, Sensitivity, Use History, Uncertainty

Administration of excess testosterone or its active metabolites appears to have little effect in the adult bird. The one notable exception is the lack of response of birds to increasing day length for the spring reproductive season, for example. Nevertheless, several chemicals have been investigated to determine whether a xenobiotic substance will introduce such a response. Antiandrogenic effects appear to be somewhat more pronounced, although none was of the same magnitude as those induced by estrogenic substances. Most of the notable changes in birds exposed to antiandrogenic substances occurred in reproductive behaviors. This lack of responsiveness in the adult bird to testosterone manipulations is not surprising, because sufficient hormonal differentiation occurs *in ovo* and during the first two posthatch weeks to maintain gender-appropriate behaviors (Hutchinson 1978). Altered male behaviors in birds result from the lack of testosterone, not elevated levels of androgens.

9.2 Juvenile Life Stages

9.2.1 Sensitivity to Androgenic Steroid Exposure

The inactive testosterone metabolite, 5β-dihydrotestosterone, had no demasculinizing effects if administered on Day 9 of embryonic development (Schumacher et al. 1989), indicating that it has no estrogenic activity. Similarly, no behavioral effects occurred in the adult bird as a result of embryonic exposure to 5β-DHT. Thus, this hormone appears to be inactive in quail. Furthermore, it is not capable of being acted upon by aromatase to form estrogens. Embryonic quail have high 5β-reductase activity in the hypothalamus, suggesting that they transform testosterone into this inactive metabolite so it cannot be aromatized into estrogen during embryonic development. Thus, although 5β-DHT is categorized as a testosterone, it has no activity, and therefore is neither an agonist nor antagonist. TCDD increases testosterone hydroxylation in herons, with the position on the ring that is hydroxylated dependent upon age and sex (Dawson 2000). However, treatment with TCDD sufficient to cause P450 induction had no effect on circulating testosterone or estradiol concentrations, suggesting that changes in hydroxylase activity were compensated for by endogenous feedback mechanisms.

Plants produce androgenic substances as well as phytoestrogens. The ketone fraction of both wheat germ oil and gibberellic acid (a plant hormone) is androgenic, as evidenced by a positive response, increased weight, in the chick comb bioassay in male chickens treated by injection

from 1 to 35 days of age (Gawienowski et al. 1997). Similar treatment with these two substances had no effect on female chickens, suggesting that their potency is relatively weak.

Hutchison (1978) showed that exposure of 6- to 12-h-old Japanese quail hatchlings to elevated testosterone through implants of testosterone proprionate had no effect on the differentiation of sexual behavior in intact males or females. In gonadectomized birds, 57% of the testosterone-treated females displayed male sexual behaviors, male vocalizations, and cloacal gland development. Gonadectomized birds that had initially been treated with estradiol benzoate were later treated with testosterone proprionate, and vice versa. Evidence suggests that a critical period during embryonic development occurs when estrogen eliminates the development of male behaviors, which normally occurs only in females. However, estrogen must continue to be present in the posthatch period for such behaviors to become established in females as adults. Estrogen given in adulthood to males castrated at hatching induces both male and female behaviors, whereas estrogen given embryonically demasculinizes males.

9.2.2 Antiandrogens

Juvenile Japanese quail exposed to aflatoxins prior to and during sexual maturation developed testes approximately half the size of testes from untreated birds. They had delayed testicular development and reduced production of testosterone (Doer and Ottinger 1980). Vinclozolin ((R.S.)-3- (3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3- oxazolidine-2,4-dione), a dicarboximide fungicide applied on crops and ornamental plants, has known antiandrogenic properties in mammals. Similar studies were conducted with Japanese quail (McGary et al. 2001). Embryos exposed on Day 4 of incubation metabolized vinclozolin to its metabolically active form, which significantly altered GnRH levels in male hatchlings, but not in females. The onset of male reproductive behaviors seemed to be delayed, with the number of mounts and cloacal contacts significantly lower at the beginning of the reproduction period. There were no measurable effects on GnRH levels in adults, plasma steroid levels in either hatchlings or adults, proctodeal foam gland growth during maturation, or relative testicular weight at 7 weeks of age. The authors speculated that observed effects were due to vinclozolin acting as an androgen receptor antagonist in the hypophyseal region of the brain.

9.2.3 Relevance, Sensitivity, Use History, Uncertainty

There has been little interest among researchers to study the potential consequences of exposing avian embryos or hatchlings to elevated testosterone levels. Hutchison (1978) and others clearly established that estrogen is the active hormone during embryogenesis, with testosterone levels remaining similar in both sexes. However, because testosterone is responsible for inducing development of singing centers in the brain, male copulatory behaviors, and the cloacal and foam glands, chemicals that might inhibit the action of testosterone should be of more concern. Unfortunately, there are few studies of the effects of antiandrogens on the developing embryo or hatchling. McGary et al. (2001) produced the most definitive study conducted to date (see Section 9.2.2). Further work needs to be done to investigate whether or not reduced levels of the 5α-metabolites are more likely to cause adverse effects through blockage of the reductase

enzymes than is reduction of the parent compound. However, there likely will continue to be less interest in understanding the potential for xenobiotics to influence the androgen system in birds than there is for the estrogen-dependent responses, due to the requirement of estrogen for demasculinization of genotypic females. This is significantly different from effects in mammals, where masculinization of the female fetus is of much greater concern, due to the requirement for elevated testosterone for genotypic males to develop normally; females will be masculinized under these conditions regardless of endogenous estrogen production. Thus, results from mammalian studies designed to determine endocrine-disrupting effects are likely not directly applicable to the avian model.

10.0 RESPONSE TO THYROID AGONISTS AND ANTAGONISTS

Relatively little work has been done to assess the effects of xenobiotics on thyroid activity in birds; only the potential for thyroid inhibition has been studied. However, hyperthyroidism can result after the cessation of treatment by thyroid inhibitors (Peebles and Marks 1991). Thyroid function can be affected as a result of interference with pituitary production of TSH, iodination and coupling of tyrosine residues to form T4, or conversion of T4 into T3 through enzymatic activation in the liver. Metabolism and excretion of T4 and T3 also could be affected, and it is possible that chemicals bind to T3 cellular receptors, resulting in either agonistic or antagonistic effects.

10.1 Sensitivity to Thyroid Stimulation

Various crude oils were shown to affect thyroid function, as measured by reduced body weights, thyroid hypertrophy, and increased plasma thyroxine levels in several species of seabirds: herring gulls, black guillemots (*Cepphus grylle*) and adult Leach's petrels (*Oceanodroma leucorhoa*) (Peakall et al. 1981). Several other hormones, such as circulating corticosterone, and corticotrophic hormone, also were elevated. The authors noted that the effects of thyroxine on regulating basal metabolism of birds are equivocal. Growth in several bird species following thyroxine administration ranged from none to moderate, with some studies indicating significant increases in body weight at low administered doses, and reduced body weight at high levels. Peakall et al. (1981) suggested that the observed effect of oil on circulating thyroid hormones most likely is a compensatory response to changes in osmoregulation induced by alterations in the intestinal mucosa due to oil ingestion, which affects both osmotic balance and nutrient uptake. This indicates that crude oil would not be classified as an EDC, because there was no direct effect of the substance on the thyroid or steroid reproductive hormone systems.

There were no other reports found in the literature documenting effects of thyroid stimulation in birds, either through direct exposure to thyroid hormone treatment or through exogenous thyroid agonists.

10.2 Inhibition of Thyroid Function

It has been suggested that maternal thyroid activity influences functional eggshell properties in birds, playing a significant role in egg hatchability (Peebles and Marks 1991). Exposure of high growth rate Japanese quail to low amounts (0.2%) of dietary propylthiouracil, a known thyroid inhibitor, had no effect on embryonic growth, but resulted in significantly reduced body water due to changes in eggshell permeability (Peebles and Marks 1991). Although molt and plumage characteristics are thought to be primarily controlled by testosterone (Stoehr and Hill 2001), thyroid hormone may play a role as well. Propylthiouracil treatment of American kestrels (*Falco sparverius*) resulted in changes in the width of subterminal bands on tail feathers, feather reflectance, and duration of the molt (Quinn et al. 2002). However, the potential for xenobiotics to result in similar responses is unclear. PCBs appear to have a bimodal response on the thyroid system: low levels are stimulatory, and higher concentrations result in inhibition (Quinn et al. 2002). Nevertheless, when kestrels were fed Aroclor 1242 at concentrations that significantly decreased circulating thyroid hormone levels, no measurable change in plumage characteristics resulted (Quinn et al. 2002). High dietary concentrations of DDT, toxaphene, and PCBs all resulted in enlarged thyroid glands and reduced body weights of adult bobwhite quail after 4 months of continuous exposure (Hurst et al. 1974). Low doses of PCBs reduced the size of the thyroid, whereas similar doses of DDT and toxaphene had no effect. The authors suggested that DDT and toxaphene could decrease the effective level of T3, thus enhancing TSH secretion, causing the thyroid to grow in size. DDT could affect T3 through changes in liver metabolic enzymes or through direct action on pituitary secretion of TSH. The authors provided no explanation for the bimodal response to PCB exposure. Grässle and Biessmann (1982) also studied the effects of DDT and PCBs on quail thyroid systems. They showed that Japanese quail exposed to either the OC, DDT, or the PCB, Aroclor 1254, for up to 120 days had significantly decreased T4 levels and altered thyroid histology suggestive of glandular inactivity, but little to no change in circulating T3 levels. The authors suggested that PCBs have a different mode of action than does propylthiouracil, possibly acting on the hypothalamus-pituitary-thyroid axis rather than at the glandular level. Furthermore, there was no relationship between T4 or T3 levels and eggshell breaking-strength for either OC or propylthiouracil exposed birds, supporting the conclusion that thyroid hormone activity does not play a significant role in reducing eggshell quality.

Dawson (2000) reviewed the effects of various OCs on growth and other thyroid functions of young birds. Such effects were first investigated in the late 1960s and early 1970s in pigeons exposed to DDT and dieldrin, then in gulls (*Larus* sp.) and pigeon guillemots (*Cepphus columba*) exposed to PCBs. Changes in mass and histology of the thyroid were measured, but were not sufficiently severe to result in gross physiological effects. Mallards (*Anas platyrhynchos*) exposed to PCBs for 5 weeks in a repeated dose exposure showed a slight increase in T3 at doses 1000 times that used in rodent studies (Fowles et al. 1997). Three-week old herring gulls (*Larus argentatus)* exposed to tetrachlorobenzene showed no changes in T4, but significantly decreased levels of T3. Conversely, *in ovo* exposure of chickens to tetrachlorobenzene showed no change in T3 and an increase in T4. *In ovo* exposure of chickens, pigeons, and great blue herons (*Ardea herodias*) to TCDD resulted in no effects on T3 or T4 levels, even when EROD and P450 enzymes were substantially induced. Field studies of

thyroid-related effects due to exposure of birds to PAHs were also inconclusive, because eggs were contaminated with both PCBs and polychlorinated dibenzofurans. Nevertheless, no significant changes in circulating levels of T3 or T4 were documented.

10.3 Relevance, Sensitivity, Use History, Uncertainty

There has been relatively little interest in studying the potential effects of xenobiotic chemicals on the avian thyroid system. Early studies were conducted to determine whether the thyroid played a significant role in eggshell quality, but as it became clear that other hormones or cellular mediators , such as prostaglandins, were more directly related to eggshell synthesis, interest waned. Effects of thyroid function on metabolic rate, such as weight gain in embryos or juveniles, was studied briefly in the poultry science field, but again did not appear to play as significant a role as did other dietary parameters. More recently, the role of thyroid hormone in plumage development was investigated, but also shown to be equivocal. It may be that the thyroid system is relatively robust and not particularly sensitive to xenobiotic effects. Generally, there is an excess of circulating T4, and approximately 30% to 40% of normal T4 levels are sufficient to produce an adequate amount of T3. Therefore, unless a chemical acts directly on conversion of T4 to T3, or binds to T3 receptors, sufficiently high doses would be required that systemic toxicity is likely to result prior to the onset of signs of thyroid dysfunction. To date, only propylthiouracil, which is a known thyroid inhibitor that is used pharmacologically to control Graves disease in humans, is known to inhibit the conversion of T4 to T3. Propylthiouracil, along with several other pharmacological agents, also inhibits the action of thyroid peroxidase, thereby reducing the amount of T4 produced.

Measurement of thyroid-specific endpoints is relatively simple, because T4 and T3 hormone structures are well conserved across species, and therefore circulating levels are easily measured with commercially available ELISA kits. Thyroid weights also can be measured at necropsy, and body weights –especially weight gain in juveniles–are routinely measured in all avian studies. Therefore, identification of thyroidogenic modes of action of xenobiotics can readily be detected during the avian two-generation test. It is likely, however, that thyroid-related fitness endpoints will be much less sensitive than other hormone-related effects and can occur at exposure concentrations that also result in generalized systemic toxicity.

11.0 CANDIDATE PROTOCOLS

Candidate protocols for the development of an avian two-generation toxicity test guidelines include established life cycle ("one-generation") reproduction tests (ASTM, OECD, and EPA) and a proposed short-term life cycle test using proven breeders (OECD 2000). Two principal designs under consideration for an avian two-generation reproduction test are a *proven breeder design* and a *pre-egg-laying exposure design*, based on a draft guideline proposed by OECD (1999) and suggested modifications of the EPA OPPTS 850.2300 one-generation avian

reproduction test guideline (EPA 1996) proposed by EDSTAC (EPA 1998) for the development of a two-generation guideline with endocrine endpoints from which the OECD (1999) proposed guideline, in part, evolved. Each of these existing and proposed guidelines is reviewed and their strengths and weaknesses discussed and contrasted. Table 11-1 compares these guidelines by test species, husbandry, test procedures, test design, endpoints, and reportable results.

Detailed reviews of the one-generation avian reproductive test guidelines and their strengths and weakness were presented by Bennett and Ganio (1991) and OECD (1996). The advantages and disadvantages of design and endpoint components of the draft OECD guideline for a two-generation avian reproduction test were reviewed in detail by Bennett et al. (2001). One-generation reproduction tests are conducted with bobwhite and mallard ducks. ASTM also permits other nonpasserine species, and OECD allows the use of the Japanese quail. The proposed two-generation guidelines limit the tests to the two quail species.

Table 11-1. Comparison of Avian Reproductive Toxicity Tests

PARAMETER	OECD Revised Draft Guideline, April 2000 Proposal for a New Avian Reproduction Toxicity Test in Japanese Quail or Northern Bobwhite Short-Term Life Cycle Test (Proven Breeders)	ASTM E1062-86 Standard Practice for Conducting Reproductive Studies with Avian Species[a] Life Cycle Test	OECD 206 Avian Reproduction Test Life Cycle Test	EPA OPPTS 850.2300 Avian Reproductive Test [e] Life Cycle Test	OECD First Draft Guideline, December 1999 Proposal for a New Avian Reproduction Toxicity Test in Japanese Quail Two-Generation Test (P1 Proven Breeders)
TEST ANIMALS					
Species	JQ, BW	Bobwhite (BW[b]), mallard duck (MD[c]), other nonpasserines	Japanese quail (JQ[d]), BW, MD	BW, MD	JQ
Age	Approaching first breeding season; Successful fertilization should have taken place before start of pretreatment BW: ≥6 months old at onset of lay	Birds of similar age; age in first season must be ±10% of mean age of group; if proven breeders used, must be same age in years	BW: 20-24 weeks ±1 week JQ: proven breeders ±½ week MD: 9-12 months ±2 week	≥7 months old; birds approaching first breeding season; same age ±1 month	Approaching first breeding season (4 wk old)
Criteria for use	Birds should appear healthy and free of abnormalities or injury that could affect test results Birds should not receive medication beginning 1 week prior to start of acclimation Successful fertilization demonstrated prior to test Parental mortality during last 2 weeks of acclimation should not exceed 3% All birds should be from same hatch	Birds from one source and strain. Birds in poor physical condition, deformed, or with plumage that differs from that of wild birds must not be used	Birds from the same population of well-known parentage Free of disease and injury Population should not be used if >3% of either sex die or become debilitated during acclimation period	Birds from same source and strain with known breeding histories, lighting regimes, disease record, drug or medication administered Not selected for resistance to toxic substances Not used in a previous test Phenotypically indistinguishable from wild stock	Birds should appear healthy and free of abnormalities or injury that could affect test results Birds should not receive medication beginning 1 week prior to start of acclimation Onset of egg laying should have taken place before start of treatment Parental mortality during last 2 weeks of acclimation should not exceed 3% All birds should be from same hatch

PARAMETER	OECD Revised Draft Guideline, April 2000 Proposal for a New Avian Reproduction Toxicity Test in Japanese Quail or Northern Bobwhite Short-Term Life Cycle Test (Proven Breeders)	ASTM E1062-86 Standard Practice for Conducting Reproductive Studies with Avian Species[a] Life Cycle Test	OECD 206 Avian Reproduction Test Life Cycle Test	EPA OPPTS 850.2300 Avian Reproductive Test [e] Life Cycle Test	OECD First Draft Guideline, December 1999 Proposal for a New Avian Reproduction Toxicity Test in Japanese Quail Two-Generation Test (P1 Proven Breeders)
One-Generation Test Guidelines					
HOUSING					
Location	indoors	Indoors; outdoors for some species	Indoors preferred; outdoors permissible	indoors	indoors
Adults	in pairs (1M:1F) To minimize injury, etc., from aggression, males and females may be housed separately; pairs placed together for ≥1 H/day for 5 days/week to maintain fertility	In pairs (1M:1F) or in groups containing no more than 1M	In pairs (1M:1F) or in groups: BW/JQ: 1M/2F MD: 1M:3F	BW: in pairs (1M:1F) or in groups (1M:2F) MD: in pairs (1M:1F) or in groups (1M:3F)	in pairs (1M:1F) by lineage, males from one line paired with females from another line To minimize injury, etc., from aggression, males and females may be housed separately; and house together long enough to maintain fertility
Young	In groups preferably by treatment group	Not specified	In groups by pen of origin Together if birds individually marked	In groups by pen of origin Together if birds individually marked	In groups preferably by treatment group F1 cohort for breeding paired (1M:F) at 4 wk old

PARAMETER	OECD Revised Draft Guideline, April 2000 Proposal for a New Avian Reproduction Toxicity Test in Japanese Quail or Northern Bobwhite Short-Term Life Cycle Test (Proven Breeders)	ASTM E1062-86 Standard Practice for Conducting Reproductive Studies with Avian Species[a] Life Cycle Test	OECD 206 Avian Reproduction Test Life Cycle Test	EPA OPPTS 850.2300 Avian Reproductive Test [e] Life Cycle Test	OECD First Draft Guideline, December 1999 Proposal for a New Avian Reproduction Toxicity Test in Japanese Quail Two-Generation Test (P1 Proven Breeders)
Pens Size (cm^2/bird)	BW: 625 JQ: 1 week old: 50 2 week old: 75 3-4 week old: 100 > 4 week old: 625	Adequate size From literature: BW: >760 JQ: >200 MD: >5000	BW: 1250 JQ: 750 MD: 5000	BW: >5000 MD: >10,000	JQ 1 week old: 50 2 week old: 75 3-4 week old: 100 > 4 week old: 625
Construction	Stainless or galvanized steel or other inert materials. Wire pens with slanting floors and egg-catchers recommended for adults. Food troughs should be covered with wire grid to minimize food spillage	Stainless steel, galvanized steel, and material coated with perfluourocarbon plastics preferred. Any nontoxic material not capable of excessive sorption of test substance, not dissolved by water or loosened by pecking are acceptable	Not specified	Galvanized or stainless steel sheeting for common walls and ceilings, and wire mesh for floors and external walls. Material coated with perfluorocarbon plastics also acceptable	Stainless or galvanized steel or other inert materials. Wire pens with slanting floors and egg-catchers recommended for adults. Measures should be taken to reduce food spillage (e.g., cover food troughs with wire grid)
ENVIRONMENT					
Temperature (°C) Adult Egg Storage	16-27 13-16	21 12-16	22±5 BW/JQ: 15-16 MD: 14-16	21 16	16-27 13-16
Hatching Brooding	37-37.5 35-38 first week 30-35 second week 23-27 third week	39 Upper brooder temperature is species-specific; provide gradient within brooder down to 21	37.5 BW/JQ: 35-38 first week, decrease by 4-5/week MD: 32-35 first week, decrease by 3-4/week	37.5 35 to 22 temperature gradient within brooder measured at 2.5-4 cm above floor	37-37.5 35-38 first week 30-35 second week 23-27 third week 16-27 fourth week on

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Percentage RH (%) Adult	40-80	45-70, higher for MD at high elevations	50-75	55	40-80
Egg storage	55-75	65	BW: 50-65 JQ: 50–70	55-80	55-75
Incubation	50-70		MD: 60-65 BW/JQ: 70-75		50-70
Hatching	70-75	70	MD: 75-85 BW/JQ: 50-75	70	70-75
Young	40-80	45-70, higher for MD	MD: 60-75 BW/JQ: 55-75 MD: 60-85	45-70, higher for MD	40-80
Lighting Intensity (Lux)	Sunlight spectrum ≥10	Sunlight spectrum 65		65	Daylight visual spectrum automatically controlled ≥10 at level of feeder
Adult (h of light)	16-17	8 prior to photostimulation (8 weeks) 17 thereafter	7-8 for 8 weeks 16-18 thereafter Transition dawn/dusk	7-8 for 6-8 weeks 16-17 thereafter or increased by 15 min/day from 17	16-17
Young (h of light)	JQ: 16-17	14		14; 15-30 min transition at dawn/dusk	JQ: 16-17
Ventilation	1 to 15 changes/h recommended	As in CCAC (1984, 1993)	Good ventilation	Good ventilation (rate: 4 changes/h in winter; 15/h in summer suggested)	8 to 15 changes/h recommended
Egg turning	Storage: optional Incubation: yes Hatching: no	Storage: optional	Storage: optional Incubation: yes Hatching: no	Storage: yes daily Incubation: yes	Storage: optional Incubation: yes Hatching: no

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Feed	*Ad libitum* Must meet nutrient requirements of the species Caloric and water content of diet must be reported Extra calcium added to adult diet if same diet used for chicks and adults	*Ad libitum* Any unmedicated commercial diet that meets minimum nutritional standards Description of basal diet: analyze for contaminants	*Ad libitum* Avoid use of chemicals or medication	Contaminant-free as possible (no pesticides, heavy metals)	Ad libitum Must meet nutrient requirements of JQ Caloric and water content of diet must be reported\ Sufficient space for feeding must be provided during first week after hatching so weak birds have access Extra calcium added to adult diet if same diet used for chicks and adults
Water	*Ad libitum* Sufficient space for drinking must be provided during first week after hatching so weak birds have access	*Ad libitum*	*Ad libitum*	*Ad libitum* Water bottles or automatic water devices recommended Bacitracin or one of its forms can be added to drinking water of young birds if necessary	Ad libitum Sufficient space for drinking must be provided during first week after hatching so weak birds have access
TEST PROCEDURES					
Quarantine				at least 2 weeks	
Acclimation	≥2 weeks BW: photostimulation 6 weeks prior to acclimation period	≥1 week Incompatible birds rearranged or replaced	≥2 weeks	≥2 weeks, can coincide with quarantine	≥2 weeks If necessary photostimulate birds during acclimation (onset of laying can occur during acclimation)

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Test Substance	Mix to obtain homogenous distribution in diet. Use of premix is advisable. Frequency of diet preparation chosen so degradation and volatilization of test substances ≥80% of initial concentration. Frequency of diet renewal ≤1X/day and ≥1X/week. Can keep frozen until use; Stability, homogeneity tests required	Physical, chemical, biological properties Analyses to determine stability in diet, frequency of diet preparation, homogeneity in diet	Chemical identification data; water solubility; vapor pressure; structural formula; purity; chemical stability in water, light, and diet; octanol/water partition coefficient; biodegradability Cannot use guideline for highly volatile or unstable substances Must have characteristics that allow uniform mixing in diet	Chemical name, source, composition: major ingredients and percentage of each Impurity, known physical and chemical properties (solubility, volatility, degradation rate) Use technical grade	Mix to obtain homogenous distribution in diet. Use of premix is advisable. Frequency of diet preparation chosen so degradation and volatilization of test substances ≥80% of initial concentration. Frequency of diet renewal ≤1X/day and ≥1X/week. Can keep frozen until use; Stability analyses under conditions on test prior to start or during range-finding ,verify during main test. Check stability at end of 1st feeding period and at end of last Homogeneity analysis prior to test of at 1st mix for study
Administration Route Carrier/diluent Maximum carrier in feed (%)	In the diet A vehicle of negligible toxicity, such as food or corn oil, water Acetone can be used if allowed to completely evaporate prior to feeding <2 A constant amount should be added to each test group and control diet to keep caloric value and moisture content equal between dosage groups	In the diet Solvent or other material Determine concentration, stability, homogeneity of test substance in diet Stability, volatility determine frequency of diet preparation and storage method <2	In the diet Water, corn oil Should not interfere with toxicity of test substance <2	In the diet Distilled water preferred. If not water-soluble, could dissolve in reagent grade evaporative diluent such as acetone, methylene chloride. Solvent should be completely evaporated prior to feeding Other carriers: corn oil, propylene glycol, gum arabic (arcacia) <2 Equivalent amount of diluent should be added to control diet	In the feed or water A vehicle of negligible toxicity, such as food or corn oil, water Acetone can be used if allowed to completely evaporate prior to feeding <2 A constant amount should be added to each test group and control diet to keep caloric value and moisture content equal between dosage groups

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Number of dietary concentrations/choice of concentration	One or multiple If multiple, then at least three plus control. Highest concentration should be level expected to reveal significant effects on adult health or reproductive parameters. Highest dose should not cause mortality or other severe signs of parental toxicity. If no significant reproduction effects expected at lower test concentration, then highest should be the expected concentration of the chemical in the environment. If this concentration is at lower than 1000 mg/kg diet, no need to test above 1000 mg/kg. Lowest concentration should not impact adult health or reproductive parameters Intermediate concentration should be geometrically spaced between the highest and lowest doses.	2 methods to establish treatment levels: control + 3 levels; geometrically spaced control +1,or more expected or known environmental concentration If nonstatistical, ≥1 concentration must produce effect or contain 0.1% of test material, or be 100X the highest field or measured concentration	At least 3 + control Results of a dietary LC50 test (TG 205) Highest concentration should approximate ½ of LC 10. Lower concentrations should be geometrically spaced at fractions of highest dose (e.g. 1/6 and 1/36 of the highest dose)	3 + control Higher 2 treatment concentrations will be multiples such as 5 of the lowest treatment level. The highest treatment levels usually will be below lethal levels. Highest nonlethal level is estimated from a dietary LC50. Concentrations should include an actual or expected field residue exposure level.	Not specified Choice on basis of toxicological data from range finding test, prior avian tests, and/or tests with mammalsHighest concentration should be level expected to reveal significant effects on adult health or reproductive parameters. Highest dose should not cause mortality or other severe signs of parental toxicity. If no significant reproduction effects expected at lower test concentration, then highest should be the expected concentration of the chemical in the environment with addition of 5X safety factor. Lowest concentration should not impact adult health or reproductive parametersIntermediate concentration should be geometrically spaced between the highest and lowest doses.
Minimum number of pens per concentration	Sufficient pairs, such as 20, to ensure 16 breeding pairs in the control groups during egg-laying. Penmates can be replaced during pretreatment	If statistic approach used, enough to give detection level of 25% at α=5, power=0.8 Nonstatistical approach, use ≥16	12 pairs or BW/JQ: 12 groups MD: 8 groups	BW: 12 pairs or groups, 20 pairs in control group MD: 12 pairs or 8 groups	Sufficient pairs, such as 20, to ensure 16 breeding pairs in the control groups to end of treatement period. Penmates can be replaced during pretreatment

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Treatment period prior to egg-laying (weeks)	no treatment prior to egg-laying	≥10 for persistent compounds <10 for nonpersistent; sometime during egg-laying	10-12	8-12	no treatment prior to egg-laying P1: 6 weeks after laying established F1 breeders from hatch through 6 wk post-fertility F1 chicks from hatch to termination at 14 days F2 not treated
Post egg-laying treatment period (weeks)	6 **(8)**	MD/BW: may be unnecessary to collect more eggs than would be laid in the wild with 2 clutches Terminate when control pens produce 25 eggs, or 6 weeks after 50% of control hens have laid 1 egg	8-10	8-12	At least 6
Egg collection	At least once daily for 6 weeks	Daily until control pens produce 25 eggs, or 6 weeks after 50% of control hens have laid 1 egg	Daily for last 10 weeks of treatment	Daily for last 8-10 weeks	At least once daily for 6 weeks
Eggs hatched	Entire pretreatment and treatment period	Entire egg-collection period	Entire egg-collection period	Entire egg-collection period	Entire pretreatment and treatment period
Candling	Day 0 for cracks Fertility/early embryo viability: BW: Day 11 JQ: Day 8 Embryo survival: BW: Day 20-21 JQ: Day 15-16	Day 0 for cracks At 1 and 2 weeks of age for fertility	Prior to incubation to detect cracks	Day 0 for cracks Fertility/early embryo viability: BW: Day 11 MD: Day 14 Embryo survival: BW: Day 18 MD: Day 21	Day 0 for cracks Fertility/early embryo viabilityDay 8 Embryo survival:Day 15-16

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Storage period	Maximum 1 week	Weekly	Weekly or biweekly	BW: Weekly or biweekly MD: biweekly	Maximum 1 week
Incubation period/hatch	BW: Day 20-21/Day 24-25 JQ: Day 15-16;Day 17-18		BW: Day 21/Day 23-24 JQ: Day 16/Day16-17 MD: Day 23/Day 25-27	BW: Day 21/Day 24	: Day 15-16/Day 17-18
Rearing period (days)	14	14	14	14	14 Brood from 6th wk used to establish F1 breeding pairs
ENDPOINTS					

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Recommended additional endocrine endpoints	Gross morphology and histology Size/weight of gonads, brain, thyroid, adrenals Histology of thyroid, brain, adrenals, gonads Testicular spermatid counts and morphology Gross abnormality of genital tract Cloacal gland area Plasma and fecal/urate hormones Steroid hormones, estradiol, testosterone, corticosterone, VTG (males), thyroid hormones, TSH Brain chemistry: GnRH, catecholamine, aromatase, foam gland test	Fresh egg weight Growth curve for young Behavior of young and adults Effects on multiple generations		Genetic sex ratio at hatching[e] For 12 genetic males and 12 genetic females/group at 14 days of age: presence at necropsy of structures on right side, histologically determined relative amount of cortex and medulla, and development of oocytes, serum sex steroids Both sexes at 14 days old: organ weights including brain, body weights, wing and bone length, thyroid weight, skeletal x-ray; if differences appear, then thyroid histopathology should be performed on all groups, otherwise, on high-dose and control groups only All surviving chicks at 14 days of age: subjected to visual cliff test, challenged with cold stress test, and nest attentiveness test Reproductive capability of offspring	Gross morphology and histology Size/weight oftestes Sex of F1 chicks

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RESULTS					
Validity of test (quality criteria)	Test substance concentration in diet is satisfactorily maintained and reported Losses of <20% initial concentrations acceptable. Higher loss rates must be investigated, explained ≥16breeding pairs of control birds that have produced eggs must survive to end of treatment period All control-group mortalities should be explained		In controls, hatching success for incubated eggs laid during fifth and sixth week of exposure should be ≥50% Rate of viability should remain ≥80% ≥10 breeding pairs of controls should survive until end of test	BW chick/MD duckling productivity in control groups does not average 12 or 10, respectively, 14-day-old survivors per pen over a 10-week period Average eggshell thickness in control groups is BW: <0.19 mm MD: <0.34 mm >10% of adult control birds die	Test substance concentration in diet is satisfactorily maintained and reported Losses of <20% initial concentrations acceptable. Higher loss rates must be investigated, explained ≥16breeding pairs of control birds that have produced eggs must survive to end of treatment period All control-group mortalities should be explained Parental mortality during last two weeks of acclimation should exceed 3% If control reproductive parameters do not meet typical values (provided in Annex 2), procedure and husbandry conditions should be checked for problems

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One-Generation Test Guidelines					
Treatment of data	Numerical data should be presented in tabular form, separating clearly the pretreatment and treatment data Treated groups compared with control group by methods in MacLeod (1994) Methods that compare individual performance before and during exposure using covariate analysis can be used If there appears to be delayed toxic responses after 1-2 gamete cycles, results should be evaluated in 1-2 week increments to avoid reduction in the power of the test to detect effects when responses from all treatment weeks are averaged together NOAEL (mg/kg body weight/day) should be determined for all health and reproductive parameters evaluated	Analyze continuous variables (body weight, eggshell thickness) by ANOVA or general linear models Separate means by multiple comparison procedures (e.g., Dunnett's test) Discrete variables such as count of eggs laid, cracked eggs, or 14-day-old survivors can be analyzed by contingency tables–chi-square analysis Derived variables such as 14-day-old survivors as percentage of eggs laid can be compared by ANOVA percentages should be arcsin transformed and weighted ANOVA performed	Test groups individually compared with control group by statistical procedure designed in study plan (e.g., ANOVA, NOAEL)	Experimental groups compared with controls by ANOVA Regression analysis is highly desirable if the data and number of dose levels allow Sample units are individual pens	Numerical data should be presented in tabular form All adult health data should be recorded per individual bird (food consumption per pair). Since P1 parental pair is primary statistical unit and F1 parental pair is secondary unit, all reproductive data should be related by lineage. Raw data should be reported by pen. Measurement of endpoints made on adult birds will be evaluated by comparing values obtained from birds from treated groups with values obtained from control birds. An NOEC expressed in mg/kg diet and mg/kg body weight per day should be determined for all health and reproductive parameters evaluated

a) Reapproved in 1991; discontinued in 2000.
b) BW Bobwhite (*Colinus virginianus*).
c) MD Mallard duck (*Anas platyrhynchos*).
d) JQ Japanese quail (*Coturnix japonica*).
e) EDSTAC (EPA 1998) recommended extension for a two-generation avian reproduction test using the Japanese quail and a pre-breeding exposure for the P1 generation. The additional endpoints recommended by EDSTAC for a two-generation test a summarized in bold under the "Recommended additional endocrine endpoint" section of this table in the OPPTS 850.2300 column.

11.1 Life Cycle ("One-Generation") Reproduction Tests

The three one-generation avian reproduction toxicity test guidelines considered in this discussion are ASTM E1062-86, OECD 206, and EPA OPPTS 850.2300. The last guideline, OPPTS 850.2300 (EPA 1996), represents a harmonization of previous test guidelines developed by the Office of Prevention, Pesticides and Toxic Substances, EPA. ASTM E1062-86 was established in 1986, reapproved in 1991, and discontinued in 2000. No replacement protocol has been proposed. OECD 206 is similar to the OPPTS 850.2300 guideline. A proposed short term life cycle (proven breeder) guide (OECD Revised Draft Guideline, April 2000, Proposal for a New Avian Reproduction Toxicity Test in Japanese Quail or Northern Bobwhite) is also discussed.

All of the life cycle tests were designed to be first-line screening tests for identifying potential reproductive effects in birds exposed to environmental chemicals. All use a similar array of measurement endpoints to evaluate effects of chemicals on components of reproduction that reflect the reproductive success of the female and prerecruitment survival of young. The endpoints are also selected to aid in identifying cause-and-effect relationships.

11.1.1 OPPTS 850.2300

The test simulates a chronic dietary exposure for the purpose of detecting potential long-term effects of persistent chemicals or those chemicals that are repeatedly or continuously applied to the environment. It takes into account the measured or estimated residues in the environment with the goal of determining both the highest and the lowest dietary concentration that produces an observable adverse effect on an array of reproduction measurements.

This guideline provides an excellent simulation of chronic exposure for chemicals that are present in the environment for long periods of time, either because of their refractory nature or application in the environment. It measures the potential for bioaccumulation of the test substance in tissues and its deposition into eggs and detects toxicity and reproductive injury from long-term exposure. The harmonized guideline provides statistical information and allows for application of regression analysis if the number of treatment levels and the data are adequate.

However, the 20-week continuous dietary exposure period is not realistic for most contemporary chemicals and pesticide exposure scenarios. The exposure protocol also does not account for the rapidity with which dietary exposure of chemicals can affect reproductive performance in birds and fails to trigger a test for substances that pose a risk to reproductive success from short-term exposure. In addition, the length of laying period appears to reduce statistical power, because it approaches the biological limits of some birds. It thereby introduces high variability within groups, because some birds remain in production while others no longer lay eggs.

A major weakness of the OPPTS test guideline that has become apparent as past data have been reviewed is that the statistical power of the test is low for some endpoints, making it difficult to detect effects that could be biologically significant.

Also, reliance on using estimated environmental concentrations to determine exposure concentrations, although sound from a realism perspective, is problematic when significant differences are detected in all dose groups or in none. If no effects are observed, it cannot be determined whether the dietary concentrations are truly below those causing reproductive deficits or whether effects could not be detected because of inadequate test design. There is also no information on how close the tested concentrations are to those that cause effects. In contrast, if effects are observed at all dietary concentrations, no information is obtained on the impact of lower concentrations on reproductive performance. Because of the usually limited concentration selection, there is little predictive capability in protocol design that would aid in the assessment of potential risk of a chemical for a new use at higher or lower concentrations than those originally tested.

The guideline does not evaluate exposures to life-stages other than the adult and egg. Therefore, many potential effects on reproduction may not be detected. With the exception of eggshell thickness, most of the endpoints measured are not indicators of endocrine disruption, and although the survivability of the P1 offspring is measured, their reproductive capacity is not. As a one-generation test, there is no evaluation of chemicals on the F2 progeny.

11.1.2 OECD 206

The OECD one-generation test is similar to OPPTS 850.2300 and shares many of the same strengths and weaknesses. However, it differs in three major ways from the OPPTS protocol. First, the Japanese quail is an acceptable species in the OECD 206 guideline. Use of the Japanese quail with its extended breeding period could reduce the variability in egg production and related parameters that is encountered in the OPPTS protocol, because the bobwhite and mallards approach the end of the reproductive limits after 10 weeks of egg-laying. Secondly, the OECD protocol recommends that Japanese quail be proven breeders before they are used in the test. The test begins with dietary exposure of the birds under short-day conditions (non-egg-laying) for 8 weeks prior to the initiation of a long-day photoperiod to bring the birds into breeding condition. Proven breeders are birds that were reproductively successful previously and that start the test approaching their second breeding season. Using firs-year breeders in a test in which the treatment is initiated prior to onset of breeding, as in the OPPTS guideline, does not allow for removing infertile or incompatible pairs, because the infertility could be a result of treatment. Use of the proven breeders reduces the variability and increase the power of the test. Thirdly, the OECD protocol establishes dietary treatment concentrations based on a range finding test that does not necessarily incorporate an estimated environmental concentration such that the concentrations tested are based on toxic response (½ LC10). The test is therefore less likely to produce the situation described above for the OPPTS guideline, where the lack of effects in all treatment groups cannot be attributed to concentrations truly below those causing reproductive effects, because the design of the test could have been inadequate to detect effects.

11.1.3 ASTME1062-86

The ASTM guideline was designed to be flexible in its approach to screening for reproductive effects. It could be used to duplicate the OPPTS 850.2300 test design or to create a design that attempts to overcome some of the weaknesses of the both the OPPTS and OECD 206 guidelines. Design options were provided in varying detail for testing nonpersistent chemicals, limiting collection of egg production to within the biological capacity of the species, assuring that the power of the test was adequate to detect effects, and selecting treatment concentrations linked to toxic response rather than field concentration.

Unlike the other one-generation studies, the ASTM guideline suggested that the exposure period for nonpersistent test substances may be shortened and dietary concentrations decreased to simulate declines under field conditions. However, the recommendations were vague, and no discussion or criteria for selecting one of the optional exposure scenarios were provided. Dietary exposure prior to the onset of egg production could be less than 10 weeks, or for some chemicals, exposure can be initiated during egg-laying. Although the potential benefit of using pre-exposure data as covariates for birds is increased power; the disadvantage is the inability to determine whether the chemical affected the onset of egg-laying.

Whereas the OPPTS 850.2300and OECD 206 guidelines recommend a post egg-laying exposure and an egg-collection period of 10 weeks, the ASTM protocol recommended an egg collection period for the bobwhite and mallard equivalent to laying two clutches, or 25 eggs. This egg-laying period is within the biological limits of these species and could reduce one source of variability that is unrelated to chemical treatment. However, the variability of proportional measurements based on the numbers of eggs laid could increase as a result of the reduced numbers of eggs collected. Also, the shortened egg-collection period, could be too short to detect effects that are delayed or increase in severity during treatment. For example, effects such as those on early germ cells in the Japanese quail would not be manifested until about 3 weeks after exposure is initiated. If birds were exposed prior to egg-laying, and the onset of egg-laying were delayed, the resulting delay in peak egg production would be overlooked by the shortened egg-collection period, and an overestimate of the severity of impact could be inferred.

The ASTM protocol also provides a statistical means for estimating the number of replicates needed to assure sufficient power to detect selected effects. Although useful and made readily calculable by means of an appended table, the information on the coefficients of variation for various endpoints, in particular endocrine endpoints, in the species or strain being used may not be available to the investigator.

The remaining strength of the ASTM guideline is the use of toxicity-based information to establish the treatment concentrations so as to avoid the shortcomings of using an expected field concentration criteria discussed in Section 11.1.1. The disadvantages of using toxicity-based criteria is the need for a range finding test(s) to establish the appropriate dietary concentrations and the diminished usefulness of tissue and egg residues if the test concentrations are considerably different from the expected environmental concentrations.

Other options of the ASTM protocol included the use of natural incubation and the potential to use behavioral endpoints and examine multigenerational effects. Little information is provided for these options.

11.1.4 OECD Revised Draft Proposal (April 2000) for a New Test Guideline, Avian Reproduction Toxicity Test in the Japanese Quail or Northern Bobwhite

The April 2000 revised draft proposal for a new test guideline, Avian Reproduction Toxicity Test in the Japanese Quail or Northern Bobwhite (ARTT 2000), is similar to the optional exposure scenarios of the ASTM guideline in that it has a limited exposure period. To accommodate bioaccumulating substances that may require lengthy exposure periods to achieve steady state concentrations in the test subjects, both the OECD 206 and the OPPTS 850.2300 guidelines prescribe 8 to 10-week exposures prior to egg laying and a similar exposure period after egg laying has begun. Exposure in the ARTT 2000 guideline is limited to 6 weeks after egg production has been established, limiting the applicability of the guideline to nonbioaccumulating chemicals. However, contemporary chemicals are more likely to be nonpersistent which reduces the severity of this limitation.

The short exposure period of the ARTT 2000 guideline is a result of an effort to increase the statistical power to detect treatment effects. By using pairs of birds that are proven breeders, pre-treatment differences in birds can be used to correct post-treatment effect measurements and reduce the variability within treatment groups; thereby making it more likely that treatment differences will be detected. Also contributing to reduced variability in the test is the ability to remove unfertile and incompatible pairs prior to start of treatment. The disadvantage of this approach is the inability to detect effects on reproductive maturation.

Although the OECD 206 guideline also provides for a proven breeder exposure scenario for the Japanese quail, it is able to retain the advantage of increased power from using pre-exposure data as covariates for birds while at the same time providing an extended exposure period of up 20 weeks by using birds approaching their second breeding season and reproductive data from the previous season. Increased cost, space utilization etc., is, of course, required to obtain the baseline data.

Although liver, spleen, and testes weights and descriptive records of abnormal behavior are required, this guideline lacks, as do the other one-generation tests, reproductive evaluation of the P1 offspring or endpoints that effectively detect endocrine disruption.

11.2 Two Generation Life Cycle Test

The two principal designs being considered for an avian two-generation reproduction test are a proven breeder design, where reproduction is monitored pre-exposure, and a pre-egg-laying exposure design, where effects on maturation are included. The (EPA 1998) recommendations for extending the EPA OPPTS 850.2300 one-generation avian reproduction test to a two-generation guideline included a pre-egg-laying exposure regime for the P1 generation. A proposed two-generation guideline (OECD 1999) was developed based on these

recommendations, but suggested a proven-breeder design. Bennett et al. (2001) discussed the advantages and disadvantages of proven-breeder and pre-egg-laying exposure protocols in detail.

11.2.1 Proven-Breeder Design

The proven-breeder protocol increases statistical power by 1) eliminating pre-egg-laying exposure allowing nonlayers to be removed prior to treatment so that the confounding effects of nonbreeders are eliminated, 2) introducing a pretreatment period during peak egg-laying, in which reproduction data are collected to be used as covariates, and 3) using a shorter egg-laying period (6 weeks), so that birds are laying at a more constant rate throughout the entire exposure. The shorter exposure period is more consistent with the minimal persistence and bioaccumulation characteristics of contemporary chemicals. The exposure can be lengthened somewhat to accommodate longer-lived substances, particularly if the Japanese quail is used. Japanese quail maintain a longer period of peak egg-laying than the period suggested in the protocol which is based on the bobwhite. A major improvement in this design over that of the one-generation protocols is the increased sensitivity of the test resulting from a more comprehensive evaluation of the P1 progeny and the addition of endocrine endpoints.

Although increased power to detect effects is obtained by this design, it comes at the sacrifice of information on maturation effects, such as delayed onset of egg-laying. However, these endpoints can be measured during the maturation of the F1 generation. Proven-breeders from a previous breeding cycle could also be used to reduce variability in a prebreeding exposure scenario. The retention of the same level of egg production and fertility over multiple breeding cycles in aging birds may be unlikely.

The statistical concerns that motivated the proven-breeder-design for the P1 generation cannot be eliminated in the F1 breeders, because they receive treatment throughout their life cycle. Therefore, because mortality is likely, the test is vulnerable to problems of decreased power to detect effects in the F1 reproduction measurements. Also, if delayed effects occurred, the 6-week period of data collection could be insufficient to detect the changes. Delayed onset of egg-laying in the F1 would also result in a delay of peak egg production. Egg production may not otherwise be impaired, but could appear to be so under the short exposure period of this design.

Measurement of endocrine status is significantly enhanced in this two-generation protocol. Endpoints include measures of steroid and thyroid hormones, maturation of hormone-mediated secondary sexual characteristics, organ condition ,and spermatid quality and number. The protocol is lacking somewhat in assessing endocrine impacts on F1 and F2 progeny. However, EDSTAC (EPA 1998) focuses on endpoints that evaluate effects on the 14-day-old chicks. Reproduction endpoints are similar to those of the one-generation studies.

A major weakness of both two-generation protocols is the reliance on detecting effects by comparison of means. There is potential for delayed effects in both the P1and F1 generations: that is, delayed manifestation of effects on spermatogonia for up to 3 weeks in Japanese quail and delayed recruitment of yolk to maturing oocytes in the P1 generation, and delayed onset and

peak production of eggs in the F1. Therefore, a regression approach to analyzing the data would better define the level and pattern of toxicity. Animal numbers can be reduced, because the dependence on replication is diminished in regression statistics.

11.2.2 Pre-Egg-laying Exposure Design

The pre-egg-laying protocol is similar to the proven-breeder design, except the birds are paired and exposed to the dietary treatment prior to egg-laying. Exposure prior to egg-laying could impact developing systems that would not be vulnerable in the mature reproductive animal. Such exposures could cause precocial or delayed onset of egg-laying that would not be detectable in a proven-breeder design. Considerable loss of power has been demonstrated when nonproducing pairs cannot be removed from the test and pretreatment egg production cannot be used as covariates to reduce sources of nontreatment variation (Springer and Collins 1999). It is possible that proven breeders approaching their second breeding season could be used so that exposure could begin prior to the onset of egg-laying, thus obtaining both information on pre-egg-laying effects and the statistical benefit of reducing the confounding effects of nonbreeders. However, age-effects would have to be taken into account, and the benefit of using pretreatment egg-production data is still lost by this option.

Because much of the information obtained from a two-generation reproduction toxicity test will be related to the P1 population, it is important that the reproductive homogeneity of the test population be assured. This is best attained by not only accounting for equivalent body condition (body size, weight, and health) in the test groups, but also equivalent ability to produce and fertilize eggs. One of the major weaknesses of the pre-egg-laying design is that it cannot assure a homogeneous test population from which to detect changes due to chemical and endocrine challenge.

12.0 RECOMMENDED PROTOCOL AND ADDITIONAL DATA NEEDS

12.1 Preferred Test Species

Of the few bird species available that breed successfully under laboratory conditions, the Japanese quail is the preferred test species, because it is an indeterminate egg-layer that matures much more rapidly than other species. It has both a higher rate of egg production and a longer period at which peak production is maintained than the northern bobwhite. In addition, the reproductive physiology, endocrinology, and behavior of the species are better characterized than those of the bobwhite. Spermatogenesis is most fully described in the Japanese quail, among avian species; therefore, the minimum length of exposure required for germ cell effects to be manifested extragonadally can be calculated. The cycle of the seminiferous epithelium of the bobwhite has not been determined. Also, the unique cloacal gland of the male can be used as a measure of maturity and androgen disruption, as can the sexually dimorphic plumage of the species. The Japanese quail has been used in toxicity tests and appears to be of comparable sensitivity to environmental chemicals as the northern bobwhite, though a direct comparison of their relative sensitivity to substances that disrupt reproduction and endocrine function is only

currently being assessed. Although there are problems such as strain characteristics and high sensitivity to inbreeding associated with the extensive domestication of Japanese quail, the advantages of using this species outweigh those of the less domesticated bobwhite.

12.2 Exposure Protocol

Currently, there are insufficient data to determine the combination of exposure protocols for the P1 and F1 generations that is the most robust for documenting changes in ecologically important fitness endpoints, and that at the same time is the most effective in determining mechanism of action. Therefore, a side-by-side performance evaluation of the prebreeding and proven-breeder exposure regimens, each one combined with a nontreated and a worst-case, hatch-through-egg-laying F1 exposure scenario is recommended to evaluate the sensitivity and cost-benefit of these protocols. The proven-layer exposure has advantages, because removing infertile pairs provides for the endpoints to be assessed from an established and homogeneous fecundity in the P1 generation. In particular, the primary biological endpoint of the test, the number of F2 14-day old survivors per P1 generation pair per day, can be assessed. Likewise, exposing the P1 generation after the onset of egg-laying avoids the complication of delaying maturation, thereby shifting the period of peak egg production in affected birds, in some cases beyond the exposure period. If reproduction is otherwise not affected, this asynchrony in production could result in misleading conclusions regarding egg production and associated endpoints, and greatly reduce the number of hatchlings available for assessing F1 survivorship and forming the F1 parental generation. There are also considerable statistical benefits in the proven-breeder scenario, when endpoints are evaluated by comparisons of means to the control.

The advantages of the pre-egg-laying exposure design are the ability to detect affects that alter maturation (e.g., onset of egg-laying, foam production) and to have long enough exposure that near steady state concentrations in the tissues of treated birds will be attained during the exposure period and adequate assessment of impact achieved for the more persistent, bioaccumulating substances.

Exposure of the F1 from hatch through egg-laying is recommended in semblance of a worst-case exposure, because it allows the expression of effects on susceptible growth and developmental stages, in addition to effects manifested in the P1 generation. For substances expected to cause significant juvenile mortality, a subset should be exposed from hatch through egg-laying, while another cohort is not exposed. The time of onset of egg-laying, one of the more sensitive measures of endocrine disruption, can be detected under this exposure regimen and could minimize the need to detect altered maturation using a prebreeding exposure scenario in the P1 population. However, it is not known whether the previous *in ovo* exposure of the F1 chicks would confound the interpretation of the response compared with the response measured in P1 birds that lack *in ovo* exposure.

Data on the onset of sexual maturation in the P1 generation could be obtained from the current one-generation reproduction toxicity tests (OPPTS Guidelines 850.2300; OECD Guidelines 206) at little added cost. The one-generation tests could also supply yolk-residue data that would provide information on the length of exposure needed for maximum transfer of the test substance

to the egg. Lastly, dose-response data from the one-generation tests would also greatly aid in selecting appropriate test concentrations for the multigeneration test.

12.3 Appropriateness of Reproductive Endpoints

In general, the reproductive fitness endpoints of reproductive output, developmental adequacy, and appropriate behaviors, for example, described in both the one-generation and two-generation candidate protocols, provide useful data on the reproductive function of the mated pairs. However, because the male has opportunity for multiple copulations, egg fertility is not necessarily a sensitive indicator of impact on male fecundity. Assessment of the relative fertility of the male at the onset of test is needed in a proven-layer exposure regimen to assure that the groups begin on test with equivalent reproductive capacity in males as well as females. An assay such as the sperm mobility test or sperm penetration of the perivitelline layer should be added as an inexpensive means of tracking gender-specific changes in fertility.

Valuable endpoints for evaluating effects on reproduction and endocrine system function in adults that are described in the candidate two-generation reproduction tests are

- onset of sexual maturation (first egg, foam production)
- cloacal gland area
- male copulatory behavior
- plasma and fecal/urate steroid hormones
- gross morphology and histology of specific organs, such as liver, spleen, gonads, brain, thyroid, and adrenals.

Some modification of these endpoints is recommended to reduce redundancy, increase the cost-effectiveness of the test, and provide higher-quality data. Both size and weight measurements of the organs are suggested in these protocols; however, size measurements of the organs provide little additional data at increased labor cost and are not recommended. Also, considerable timesaving will be realized during necropsy if the thyroid and adrenal glands are excised for histological examination rather than for organ weight. Removing and trimming these tissues for weight measurements is time-consuming and damaging to the tissue. Better quality data are obtained from histological examination of these tissues. Histological examinations should only be conducted on organs from birds receiving the highest dietary concentration of the test substance and on controls. If chemical-induced abnormalities are observed, the tissues from birds in the lower dose groups should be examined.

Other endpoints suggested by the candidate two-generation protocols that are not useful at this time are spermatid counts and morphology, VTG, and several brain chemistry measures. Microscopic enumeration of spermatids and morphological abnormalities of sperm are time-consuming to document and have been found to be relatively poor indicators of reproductive capability in male birds. A major effort in poultry research in recent years has been the development of more reliable assays of male condition based on measurements of sperm function, rather than semen quality. These assays are mainly based on measures of the interaction of sperm with the inner or outer perivitelline layer of the egg. They do not require

special expertise or physically handling the bird to obtain a semen sample (samples can be obtained from the same eggs as those used for egg quality tests) and are inexpensive to perform. Substitution of a perivitelline assay for the spermatid counts is recommended. It is not known at this time how useful VTG assays will be in birds, and only recently has an assay been reported that uses the necessary species-specific antibodies for quail. If it is as sensitive of an endpoint as it has been shown to be in other oviparous animals, it could be a valuable inclusion in the guideline at a future date. Several brain chemistry endpoints, such as GnRH, catecholamine, aromatase, and vasotocin, which have been used to identify and investigate hormonally dimorphic areas of the avian brain, are histochemical techniques requiring considerable expertise, preparation, and computer image analysis. It should be noted that these endpoints are currently being adapted to more routine laboratory procedures and evaluated for their potential use as indicators of endocrine disruption. Few data are available in the literature on these endpoints, because they are only currently under testing. Researchers conducting this work report that GnRH appears to be relatively insensitive as an endpoint of endocrine disruption, but that preliminary data from dose-response tests with a weak estrogenic compound indicate that catecholamines and vasotocin are promising, having application at all life-stages and adaptability to routine laboratory procedures, such as RIA. These procedures are obviously terminal, requiring the sacrifice of the animal, and would therefore be applied to brain tissue gathered at adult necropsy and from the 14-day chicks.

EDSTAC (EPA 1998) endpoint recommendations mainly focus on evaluation of the 14-day surviving chicks. Determining the genetic sex of the 14-day old survivors at hatch provides a good measure of *in ovo* disruption of endocrine systems. EDSTAC (EPA 1998) then recommends selecting a subset of males and females from each group for gross and histological examination of gonadal tissue. The gonadal tissue of all males and all females in the high-dose group and controls should be weighed and its external appearance described. Relative amount of cortex and medulla, and the development of oocytes should be histologically determined on a subset of males, as described by EDSTAC (EPA 1998). Oviduct weight and differentiation should also be determined. However, to keep costs of the test within a reasonable range, these endpoints should be measured on a single cohort of hatchlings, preferably from the latter half of the exposure period, to assure maximum transfer of the test substance to the yolk. If the incidence of phenotypic change in the high-dose group is above that found in the control group, the remaining treatment groups should be examined. Companion determination of circulating steroid concentrations is also a valuable endpoint suggested by EDSTAC (EPA 1998). The committee also suggested the determination of organ weights, particularly the thyroid weight, for all chicks. As discussed above, thyroid weights are not recommended, because of the difficulty in removing this tissue without severely affecting the more informative histological examination of the tissue. The benefit of collecting organ weights on all chicks does not appear to warrant the cost involved and is not recommended. However, the wing and bone length measurements would provide an index of thyroid function. Although skeletal x-ray would provide an additional measure of thyroid effects, it does so at high cost and also is not recommended. However, x-ray could also be used to detect medullar bone formation in males and females in response to chemical challenge. A battery of behavioral tests is recommended by EDSTAC (EPA 1998) for the 14-day-old chicks. This endpoint has merit, though the composition of the

test battery needs further investigation to refine protocols, validate sensitivity, and assure applicability to a broad range of chemicals.

12.4 Preferred Methods for Quantification of Biochemical Endpoints

The sex hormones, corticosterone, and the thyroid hormones can be determined by RIA or ELISA methods. Commercial kits are available and are adaptable to use on birds and samples from various matrices. RIA methods are often more sensitive, but ELISA techniques, also highly sensitive, lack the additional cost and issues involved with working with radioisotopes. Because many of the hormones respond rapidly to stress, handling, and bleeding procedures, the preferred sampling method is from fecal/urate samples, rather than from plasma. Fecal steroid sampling has been validated in a wide array of animals including birds, and evaluated under both laboratory and field conditions. Sampling feces will allow periodic sampling of hormone status rather than single-point, terminal evaluations that can be misleading, particularly for thyroid hormones. Steroid hormones can also be sampled during egg-laying, by this manner, without the danger of decreasing egg production or inducing body checks in eggs from handling stress of the hens. Because male Japanese quail usually must be removed from the hen several times during a week to prevent injury from aggressive behavior by either mate, monitoring of fecal steroids by sex can be accomplished in their separate home cages. Although the thyroid hormones have not been measured in feces, it is highly likely that this noninvasive method can be easily applied to T3 and T4. Use of fecal/urate samples for hormone monitoring under the dosing regime of the two-generation laboratory test will also provide a direct comparative measure for field collected samples.

Determination of genetic sex by DNA methods is preferred over the more lengthy and costly karyotyping techniques. Western Dot Blot or PCR methods are equally reliable; the dot blot technique is somewhat more rapid.

Several promising biochemical methods in development (VTG, catecholamine, and vasotocin) are being adapted to RIA or ELISA techniques because of their superior sensitivity and ease of measurement. Again, additional convenience and some cost savings are attained when ELISA techniques are employed.

12.5 Significant Data Gaps

Several data gaps were found in the course of the review. Among these gaps is the lack of clear information on the transfer to and fate of xenobiotics *in ovo*. Chemical transfer to the egg has been variably described from tissue or dietary sources. The source of the *in ovo* concentrations of test substances has impact on decisions regarding exposure duration and interpretation of endpoint responses. Further study of estrogenic xenobiotics that require metabolic activation is needed to determine whether or not this occurs in the embryo, and if so, its relationship to the sensitive developmental period. Additional work is also needed to determine whether reduced levels of the 5α-metabolites, through blockage of the reductase enzymes, are more likely to cause adverse effects than is reduction of the parent compound.

Because histopathological evaluations are observer-based, the procedures for their use must be standardized to produce repeatable results that can be verified by different investigators. Avian histopathological evaluations in the context of toxicity assessments have had little formalization of technique, description, or morphometric analysis although there are standard practices and vocabulary in use by organizations such as the American Association of Avian Veterinarians and the College of Veterinary Pathology

Little is known of the interactive effects of endocrine-active substances. In particular, most test diets (and vegetable oil carriers) contain variable amounts of the natural phytoestrogens and phytoandrogens. How these chemicals affect the bird's response to the test substance (a suspected endocrine-active compound) is unknown. Dietary treatments or changes to eliminate this potential interference will need to be investigated.

Significant genetic differences exist between strains of Japanese quail that are used in avian reproductive testing. It is not known whether these differences can have significant effects on the outcome and interpretation of the test. Further investigations into traits that are coselected with high body weight or high fecundity are needed. The feasibility and criteria for establishing and maintaining a random-bred line from a natural source, such as UBC, or a standard random-bred line with selected qualities for toxicity testing needs investigation.

If ANOVA methods continue to be applied to avian reproduction toxicity tests, a statistical approach for delayed effects must be investigated. For example, in a 6-week exposure period, only during the final 3 weeks of this period will the full effects of a test substance on fertility, embryo viability, hatching success, and sperm quantity and quality be observed because of the about 21 days needed for effects on spermatogonia to be expressed extragonadally. No effective statistical method has been identified for analyzing such a delayed treatment effect so far. Springer and Collins (1999) conducted simulation tests using an adaptation of the Roth step-down trend test incorporating covariates for the last 3 weeks (Weeks 8 -10) of a standard bobwhite quail reproduction toxicity test. They found that using only the last 3 weeks of data can result in a decline in the power of the test to detect the number of chicks that hatched per number of eggs incubated, and the number of hatchlings that survived 14 days.

Though seemingly a minor data gap, the lack of specific information on husbandry requirements of the Japanese quail that will result in consistent results in laboratory toxicity tests is important. As indicated in a comparative study of five laboratories using the same source of birds and test substances (Schlatterer et al. 1993), a number of parameters differ significantly among laboratories (e.g., number of eggs with cracks, food consumption, eggs laid, etc.), potentially resulting in different conclusions regarding the hazard a chemical poses. Species-specific information needs to be developed for dealing with fear, social stress, injurious pecking , and other behavioral problems of Japanese quail in laboratory reproduction tests. These behaviors seriously harm the birds' welfare and productivity (Jones and Hocking 1999). Much can be learned from anecdotal information and experience of flock curators, but it should be verified for the various growth and maturation stages of the quail in testing situations.

13.0 IMPLEMENTATION CONSIDERATIONS

Pre-validation considerations that are important to the development of the avian reproduction test include the following. Because there is not enough information available to determine the combination of the P1 and F1 exposure protocols that is the most advantageous for evaluating reproductive effects and determining mechanism of action, a direct performance comparison of the proven-breeder and prebreeding exposure regimens combined with nontreatment and worst-case hatch-through-egg-laying F1 exposure scenarios is needed for the selection of the appropriate exposure regimen.

Considering the sensitivity of the Japanese quail compared with that of the northern bobwhite, the effects of strain selection on test outcome should be determined prior to implementing the test protocol to verify the sensitivity of the test species and to minimize nontreatment variability across laboratories. Likewise, husbandry practices need review and standardization to reduce interlaboratory variability and to reduce behavioral problems of Japanese quail in laboratory reproduction tests.

Avian histopathological evaluations in the context of toxicity assessments have had little formalization of technique, description, or morphometric analysis. Because histopathological evaluations are observer-based, the procedures for their use must be standardized to produce repeatable results that can be verified by different investigators.

If ANOVA methods continue to be applied to avian reproduction toxicity tests, a statistical approach for delayed effects should be investigated.

T4/T3 are important indicators of thyroid function, but are limited in value when obtained from plasma samples because of their sensitivity to handling and bleeding, and because plasma fluctuation of these hormones render it difficult to document hypo- or hyperthyroidism from a single sample. These hormones are excreted through the bile. Therefore, development of T4/T3 assays in fecal/urate samples could make the ability to monitor thyroid hormones noninvasively over time readily available.

PCR methods for genetic sex determination need to be optimized for the Japanese quail.

The interactive effect of phytoestrogens in test diets needs investigation, and concentration limits for natural phytoestrogens in feed should be established relative to the potential impact of these compounds on test outcome.

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